6

BIOREACTORS FOR TISSUE ENGINEERING

Learning Objectives

After completing this chapter, students should be able to:

1. Provide examples of the role of biomechanical forces in nature.
2. Provide a definition for bioreactor.
3. Explain the difference between enabling and supporting technology for tissue engineering.
4. Describe the classification scheme for bioreactors and distinguish between bioreactors for cell culture, scaffold fabrication, tissue fabrication, and physiological conditioning.
5. Discuss design considerations for bioreactors.
6. Provide an example of an idealized bioreactor system.
7. Describe integration between tissue engineering and bioreactor technology and explain how bioreactors can be used at different stages of tissue fabrication.
8. Discuss the role of bioreactors in supporting and/or enabling fabrication of 3D artificial tissue.
9. Explain how bioreactors are used for mammalian cell culture.
10. Discuss the use of bioreactors for scaffold fabrication.

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

11. Provide examples of bioreactors used for scaffold cellularization.
12. Explain the importance of perfusion in tissue engineering.
13. Explain the importance of stretch in tissue engineering, and describe the effect of stretch on smooth muscle cells, endothelial cells, and cardiac myocytes.
14. Explain the importance of electrical stimulation in tissue engineering.
15. Provide examples of bioreactors for electrical stimulation in tissue engineering.

CHAPTER OVERVIEW

In this chapter, we will look at the critical role of bioreactors in the fabrication of artificial tissue, and integration between the fields of bioreactor technology and tissue engineering. This chapter will start with examples of biomechanical forces in nature to illustrate the importance of these forces on tissue formation and function. This will be followed with a working definition of bioreactors, along with a classification scheme for bioreactors. We will present design considerations for bioreactors and follow this up with a description of an idealized bioreactor system. The idealized system is based on our wish list for functional capabilities for bioreactor technology. We then present a scheme outlining the integration between bioreactor technology and tissue engineering and will illustrate the role of bioreactors at every stage of artificial tissue fabrication. We next provide examples of bioreactors for mammalian cell culture, scaffold fabrication, and cellularization and bioreactors for perfusion, stretch, and electrical stimulation. In each case, we provide design considerations, effects of stimuli on tissue function, and examples of specific bioreactor systems that are currently in use at research laboratories.

6.1 INTRODUCTION TO BIOREACTORS

The development of bioreactor technology is a fundamental component of tissue engineering. The field of bioreactor technology has progressed in parallel with tissue engineering, as a scientific discipline. During the early phases of tissue engineering, fabrication of artificial tissue involved culture of cells within a 3D support matrix with cell-cell interactions and cell-matrix interactions leading to development of functional 3D artificial tissue. This remains the first step for 3D artificial tissue fabrication, even in current research. However, it was not long before researchers recognized the need for bioreactor technology in the fabrication, culture, and maintenance of artificial tissue. As is often the case in tissue engineering, inspiration is drawn from nature, and the development of bioreactor technology is no exception.

During normal human function, tissues are exposed to biomechanical forces, which are important in maintaining form and function of the particular tissue. For example, the heart is a dynamic organ that continuously generates force in response

to a depolarization wave, and every time the heart beats, there is a change in the local stress environment. *What does this mean in terms of bioreactor technology?* This has clear implications in the development of artificial heart muscle, which needs to be cultured in the presence of continuous electrical stimulation and mechanical stretch, and subjected to continuous media perfusion. During the fabrication of artificial heart muscle, bioreactor technology will need to be implemented to simulate *in vivo* physiological conditions during controlled *in vitro* culture. This is the case with any tissue system under development—*in vivo* conditions need to be recapitulated *in vitro* by the development of bioreactor technology. Simply stated, bioreactor technology is important to support tissue development and maturation by simulating *in vivo* conditions during *in vitro* culture.

We can obtain a better understanding of the importance of bioreactor technology by looking at one specific example: changes in human physiology of astronauts during space missions. When astronauts are deployed to space missions, there is limited compression on their musculoskeletal systems due to the low gravity environment in space. Compression is required to maintain bone and skeletal muscle mass during normal physiological function. As a result of the low gravity environment in space, when astronauts return from space missions, they have a deficit in bone and skeletal muscle mass due to the lack of compressive forces on the musculoskeletal system. This loss of bone mass is reversible and can be recovered by physiotherapy, which involves subjecting skeletal muscle to gradually increasing compressive forces. This example shows the adaptive nature of tissue and the ability to modulate tissue function based on external stimuli: physiological signals in the human body and bioreactors in tissue engineering.

Let us look at some examples of biomechanical conditioning in nature. We have already seen that heart muscle is constantly exposed to electrical stimulation and mechanical stretch. We have also seen that bone is under constant compression and that compressive forces are important to maintain critical bone mass. Skeletal muscle is also very dynamic and an increase in muscle activity in the form of exercise results in an increase in muscle mass; the opposite is also true, as skeletal muscle undergoes atrophy in times of inactivity. Blood vessels are constantly exposed to pulsatile fluid flow on the luminal surface, and this stress environment is important for normal function.

We can view bioreactors in the context of physiological conditioning, designed to replicate and deliver specific stimuli to 3D artificial tissue at various stages of development to support formation, development, and maturation of functional tissue that is similar in form and function to mammalian tissue.

6.2 BIOREACTORS DEFINED

In the previous section, we introduced the concept of bioreactors by looking at the role of biomechanical forces in nature and the role of these forces in maintaining homeostasis during normal function. We also stated the essential role of these biomechanical forces during the fabrication and culture of artificial muscle, and

stated that bioreactors are required to achieve this objective. *But what exactly is a bioreactor and how do we define it?* Unfortunately, there is no universally accepted definition for bioreactors in the field of tissue engineering. This is due to the diversity of functions for which bioreactors are used, and the degree of customization required from one application to another. This often results in different views on the definition and applications of bioreactors for tissue engineering. Although these differences exist, we believe that it is important to have a unifying definition for bioreactors.

Bioreactors have been used for a wide range of functions, including cell culture and proliferation, scaffold fabrication, artificial tissue formation, and for providing controlled physiological conditioning (mechanical stretch, electrical stimulation, continuous media perfusion) for 3D artificial tissue development and maturation. We will provide additional details on these specific applications for bioreactor technology in the next section. The use of bioreactor technology is intertwined with the process of 3D artificial tissue fabrication. Based on the specific application, bioreactors have either been categorized as enabling technology or supporting technology in tissue engineering. Enabling technology refers to any process that "enables" the formation of 3D artificial tissue; such technology is used at any stage prior to the fabrication of 3D artificial tissue. Enabling technology for bioreactors refers to systems that support cell culture and proliferation, scaffold fabrication, and tissue fabrication. In all of these cases, bioreactors are used to enable the process; they are used prior to the formation of 3D artificial tissue. Supporting technology refers to bioreactors used after formation of 3D artificial tissue; they are used to "support" the development, growth, and maturation of artificial tissue.

Since a universally accepted definition of bioreactors does not exist, we will build our own definition. We can develop a definition of bioreactors by assessing the components of the word bioreactor—"bio" and "reactor". "Bio" refers to the application of technology for biological purposes, and in tissue engineering, everything is biologically based. "Reactor" is often used in chemical engineering and refers to specific devices that are used for a chemical reaction. Bioreactors are generally not used to support any chemical reactions or reactions of any other sort during the fabrication and/or culture of artificial tissue. The use of the term "reactor" is not directly linked to bioreactor applications in tissue engineering, but due to its extensive use in the literature, the term will be retained.

What have we learned thus far about bioreactors that can assist in developing a working definition? Bioreactors are used extensively in tissue engineering for all steps in the tissue fabrication process, they are considered either enabling or supporting technologies, and in most cases, they do not involve chemical reactions. Based on this framework, we propose the following definition for bioreactors:

"Bioreactors are devices used extensively in tissue engineering to *enable* the fabrication of artificial of 3D artificial tissue and *support* the growth, maturation, and development of artificial tissue during controlled *in vitro* culture." This definition encompasses the breadth of applications for bioreactors in tissue engineering and embodies the use of bioreactors as enabling and supporting technologies. This is illustrated in Figure 6.1.

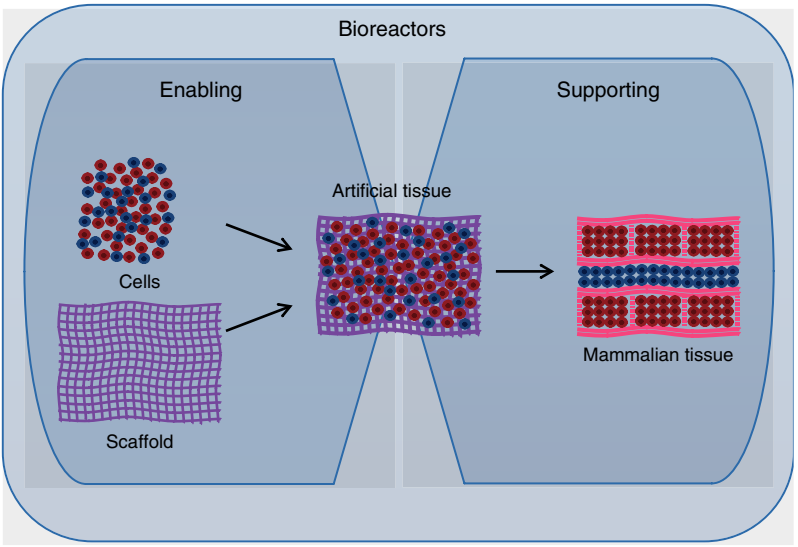

Figure 6.1 Definition of Bioreactors–Enabling—Technology required for the fabrication of artificial tissue. **Supporting**—Technology required for development and maturation of 3D artificial tissue. **Bioreactors = Enabling + Supporting**.

6.3 CLASSIFICATION OF BIOREACTORS

We introduced the concept of bioreactors in the first section by looking at biomechanical forces in nature and the need to simulate these forces during fabrication and culture of artificial tissue. In the previous section, we provided a unifying definition of bioreactors and described many applications of bioreactor technology in tissue engineering, including cell culture, scaffold fabrication, scaffold cellularization, and bioreactors for stretch, perfusion, and electrical stimulation. In this section, we describe each of these and provide a classification scheme for bioreactors.

Bioreactors for Cell Culture and Expansion—A large number of cells are required for most tissue engineering experiments. Cells are cultured in tissue culture plates and/or flasks, and once the cellsbecome confluent, they are subpassaged and cultured on additional tissue culture plates to increase cell yield. This process is done manually and has several limitations. Culture and expansion of cells is expensive and time-consuming, and working with a large number of culture plates can increase the risk of contamination. Many of the limitations of traditional cell culture can be overcome by using automated systems to perform all the tasks associated with the culture and expansion of mammalian cells (1–6). Cell culture bioreactors have the capacity to perform all tasks associated with the maintenance of cells, including media changes and supplementation of the media with growth factors and cytokines (Figure 6.2a). These bioreactors can also undertake all tasks

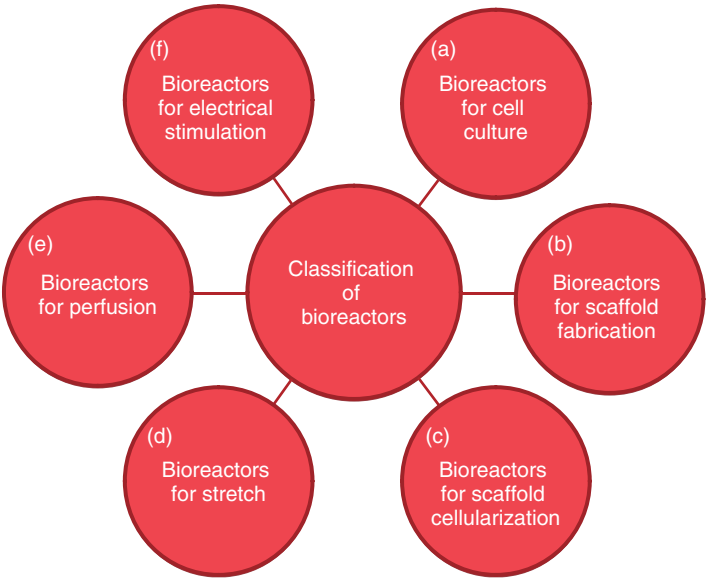

Figure 6.2 Classification of Bioreactors—**(a) Bioreactors for Cell Culture**—Robotic nozzles are used for delivery and aspiration of cell culture media. **(b) Bioreactors for Scaffold Fabrication**—Electrospinningis used for scaffold fabrication and leads to the formation of nanofibers, which can be assembled to form porous 3D scaffolds. **(c) Bioreactors for Scaffold Cellularization**—The cell suspension is perfused through a porous scaffold to promote scaffold cellularization. **(d) Bioreactors for Stretch**—Artificial tissue is subjected to continuous cyclic strain. **(e) Bioreactors for Perfusion**—Input and output ports are engineered within the culture vessel to support continuous media perfusion. **(f) Bioreactors for Electrical Stimulation**—Electrodes are engineered within the culture vessel to deliver controlled electrical stimulation.

associated with subpassaging, including trypsinization, dilution, and replating of these cells. All processes take place under sterile conditions using robotic arms and guidance control via software algorithms. The use of these bioreactors minimizes human effort and reduces the probability of contamination.

Bioreactors for Scaffold Fabrication—Electrospinning has been used extensively for the fabrication of scaffolds with very tightly regulated fiber architecture and diameter, ranging from nanometer to micrometer size (7–12). During the electrospinning process, the polymer is first solubilized in a suitable solvent and then, in response to a high voltage electric charge, discharged as a fluid jet. This fluid jet travels in air. During this process, the solvent in which the polymer is solubilized evaporates, leaving behind a thin microfibrous structure. The thin fibers are collected in a mandrel or some other collection device and are used to form complex scaffolds with individual fibers as the starting material (Figure 6.2b). This process has resulted in the fabrication of very complex scaffolds, which have been used to support a wide variety of tissue engineering applications.

Bioreactors for Scaffold Cellularization—This category of bioreactors is designed to support the fabrication of 3D artificial tissue in a controlled sterile environment. One of the steps in the process of tissue fabrication involves cellularization of 3D scaffolds. Scaffold cellularization can be achieved manually by suspending isolated cells in culture media and injecting the cells into the 3D scaffold using a syringe. However, direct cell injection does not result in a uniform cell distribution throughout the scaffold; it is an imprecise process and very user-dependent. In order to circumvent this problem, bioreactors have been developed to promote scaffold cellularization using many different techniques, including perfusion based technologies (13) (Figure 6.2c). In the case of perfusion bioreactors, cells are suspended in culture media and then perfused through a porous scaffold; perfusion seeding of cells results in uniform cell distribution throughout the 3D scaffold.

During normal human function and development, all cells and tissues are constantly exposed to a myriad of signals that guide tissue maturation and development, and alter tissue function. These signals are in the form of electrical currents, mechanical stretch, compression, fluid shear stress, and changes in the chemical environment (14–17). Bioreactors for stretch, perfusion, and electrical stimulation are designed to recapitulate *in vivo* stimulation protocols during *in vitro* culture and maturation of 3D artificial tissue. Collectively, this group of bioreactors, designed to deliver controlled signals for physiological conditioning, are the largest group of bioreactors in tissue engineering.

Bioreactors for Stretch—Stretch is an important modulator of tissue function, particularly for muscle tissue. During normal function of mammalian cardiac muscle, cells are exposed to repetitive cycles of lengthening and shortening; this stimulation is important for normal cardiac function. Bioreactors for stretch are designed to replicate these continuous cycles of lengthening and shortening of muscle tissue, and to support the development and maturation of artificial tissue (Figure 6.2d).

Bioreactors for Perfusion—In the human body, the circulatory system provides a distribution network for blood flow and serves to deliver nutrients to all cells and tissue and remove waste products. All cells in the human body are located within a couple hundred microns to a capillary; this property is critical to support cell and tissue viability. Bioreactors for perfusion are designed to do the same thing for 3D artificial tissue by providing continuous flow of cell culture media to deliver nutrients to artificial tissue and remove metabolic waste products (Figure 6.2e).

Bioreactors for Electrical Stimulation—During normal function of excitable tissue like cardiac muscle, skeletal muscle, and neural tissue, changes in voltage play an important role in maintaining and supporting cell and tissue level function. As an example, depolarization waves in the heart are due to voltage changes, which in turn provide the trigger for cardiac muscle contraction. Bioreactors for electrical stimulation are designed to replicate these changes in voltage and other electrical properties observed during normal mammalian function to support the growth and development of artificial tissue (Figure 6.2f).

6.4 DESIGN CONSIDERATIONS

The process flow chart for bioreactor design is presented in Figure 6.3 and consists of four steps: 1) definition of stimuli, 2) control of processing variables, 3) sensor technology, and 4) stimulation protocol. Let us look at each of these in more detail.

Definition of Stimuli—The most important design consideration that needs to be addressed during bioreactor design and fabrication is the stimuli that needs to be delivered to the cells and/or tissue. All other steps in the design process revolve around the nature of the stimuli. In most cases, the objective is to recapitulate *in vivo* conditions *in vitro*, and this is particularly the case when designing bioreactors for cell culture/proliferation and physiological stimulation of 3D artificial tissue. Some examples of stimuli that are used in bioreactors include mechanical stretch, electrical stimulation, continuous media perfusion, and compressive forces. The specific stimulation will depend on the type of tissue being fabricated, with compressive forces being important for bone tissue and electrical stimulation and stretch being important parameters for artificial heart muscle.

Control of Culture Variables—During culture of artificial tissue using bioreactor stimulation, processing variables need to be regulated. These culture variables include temperature, pH, and composition of the gaseous environment. The can be done in one of two ways. In the first case, the bioreactor and 3D artificial tissue are all maintained in a cell culture incubator, which has all the necessary mechanisms in place for control of culture variables. This makes use of a commercially available system, which has been tested and validated, to control the culture environment. This process also simplifies bioreactor design and fabrication considerably. The second strategy is to engineer control systems within the bioreactor system. This is a complex process, requires advanced engineering and sensor technology, and increases the time and cost of bioreactor development. However, in-house control of the culture environment provides a high degree of process control, something which is not available in a cell culture incubator.

In the case of in-house regulation of processing variables, temperature regulation has been achieved by heat exchange from water-jacketed chambers or by use of heated metal plates with feedback control. The regulation of pH often involves injection of CO_2 into the culture environment with feedback control to regulate pH at 7.4 and CO_2 at 5%, similar to what is seen in a cell culture environment. A variety of buffers are also available for pH regulation.

A balanced strategy could involve building a first-generation bioreactor that is used in a cell culture incubator; this can be used to test and validate bioreactor functionality. Once successful, a second-generation system can be developed to incorporate control of processing variables in the environment; this way, bioreactor development has been completed in an earlier phase.

Sensor Technology—Another important design consideration is sensor technology, also one of the least developed technologies in the bioreactor field. There are two categories of sensor technology. First, sensors are needed to monitor environmental conditions like temperature, pH, and gaseous environment and

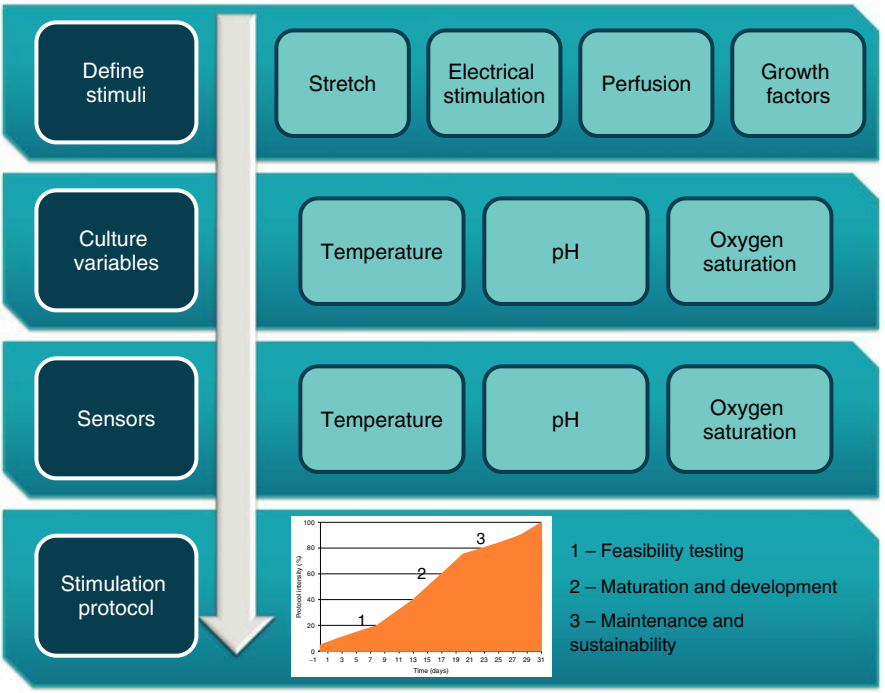

Figure 6.3 Design Consideration for Bioreactors—The flow chart outlines a decision-making process for bioreactor fabrication. **(a) Define Stimuli**—The first step in the process requires identification of specific stimuli to be delivered to 3D artificial tissue. Specific stimuli could be stretch, electrical stimulation, perfusion, and/or compression. **(b) Culture Variables**—During culture of artificial tissue, processing variables need to be regulated. These include temperature, pH, and gaseous composition. Control of processing variables can be accomplished in one of two ways. The bioreactor can be placed inside of a cell culture incubator, which has the capability to control these processing variables. Alternatively, bioreactors can be engineered with these capabilities; in this case, the bioreactor can be operated independent of a cell culture incubator. **(c) Sensors**—Sensor technology is required to monitor culture variables and to measure changes in the functional performance of 3D artificial tissue over time. There are two categories of sensors. First, there are sensors to monitor culture variables like temperature, pH, and gaseous composition, and second, there are sensors to monitor tissue function. **(d) Stimulation Protocol**—The final decision that needs to be made is the specific stimulation protocol to be used to condition 3D artificial tissue. The stimulation protocol is divided into three phases, which include feasibility testing, maturation and development, and finally, maintenance and sustainability.

use this information in a feedback control loop. This area of sensor technology is fairly well-established. The second area involves functional assessment of artificial tissue and again, use of this information for feedback control of tissue development. This area of sensor technology is poorly developed. The most common approach involves removing artificial tissue from the bioreactor at

regular intervals for functional assessment. At this time, artificial tissue is sacrificed and the functional data acquired is not used in a feedback control loop. In addition, sampling points are few, infrequent, and are invasive and interface with tissue function. What is required is noninvasive real time monitoring of 3D artificial tissue function and use of this information in a feedback control loop.

Stimulation Protocol—Another important variable is the stimulation protocol—for example, *if we develop bioreactors for uniaxial stretch for cardiac patches, what stretch protocol should we use?* The parameter space can be defined by stretch frequency, percentage stretch, and duration of stretch. One may be tempted to initiate a stretch protocol that replicates stretch protocols observed under normal physiological function. While this may be a reasonable starting point, it should be cautioned that artificial tissue may be at an early stage of development. This means that artificial tissue may not be able to sustain a rigorous stretch protocol; it may be better to start with a milder stretch protocol and gradually progress toward replicating harsher *in vivo* conditions. The stimulation protocol should be divided into three phases. The first phase should be designed to test the feasibility of using any specific stimuli with 3D artificial tissue and involves very low stimulation protocols, designed as initial feasibility testing. Once artificial tissue sustains a low level of stimulation, stimulation intensity can be slowly increased to support development and maturation of 3D artificial tissue; this refers to the growth and maturation phase of 3D artificial tissue. Finally, as the stimulation protocol approaches values of *in vivo* conditions, there is concern of excessive stimulation, which can lead to tissue fatigue or damage. In this final phase, any increase in stimulation intensity needs to be incremental and carefully graduated, and the tissue response must be tightly monitored.

A Final Note on Design Considerations—As we develop bioreactor technology, a critical area needs to be addressed. *In vivo*, mammalian tissue is not exposed to a single variable at any given time, but rather, it responds to a diverse array of signals; in addition, there are temporal and spatial variations in these signals. However, most bioreactors are designed to deliver a single stimuli, for example: stretch, electrical stimulation, or perfusion. Further, in most cases, there are no temporal or spatial variations in the stimulation signals. This is an area that needs to grow as the field of tissue engineering evolves, and advanced bioreactors need to be developed that have the capability of controlling multiple stimulation protocols at the same time and supporting temporal and spatial variations in signals.

6.5 IDEALIZED BIOREACTOR SYSTEM

In this section, we discuss an idealized bioreactor system for the growth and maturation of artificial heart muscle (Figure 6.4). While components of such a system have been developed, a complete bioreactor that has all of the capabilities listed here has not been fabricated. As we have seen from our discussion thus far and will see in the reminder of this chapter, technological innovation in the bioreactor

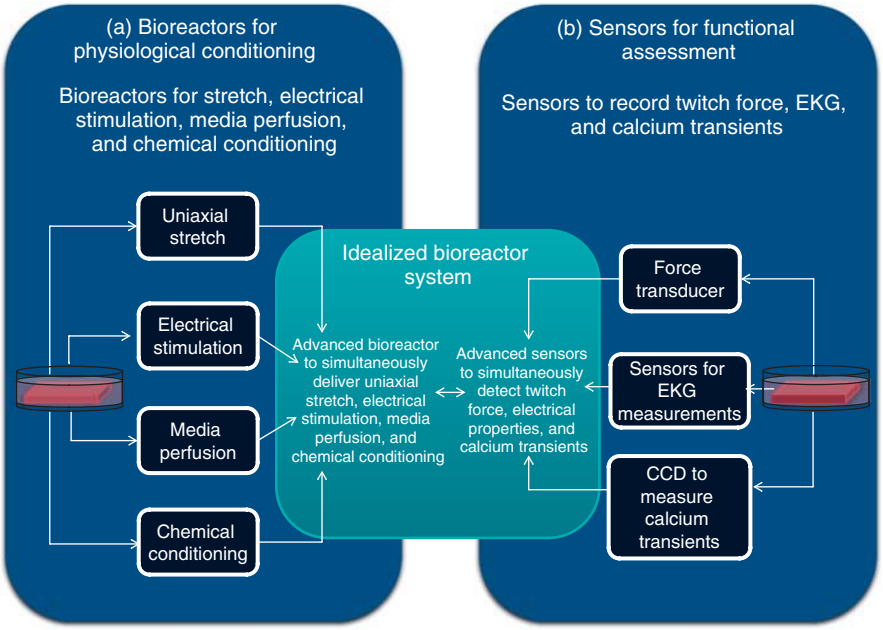

Figure 6.4 Idealized Bioreactor System for 3D Artificial Heart Muscle—(a) Bioreactors for Physiological Conditioning—Artificial heart muscle is fabricated in a tissue culture plate and stimulated using bioreactors for stretch, electrical stimulation, media perfusion, and chemical factors. In most cases, this is done using independent bioreactors for each stimuli; in the idealized bioreactor system, a single bioreactor will have the capabilities to deliver all four stimuli to condition 3D artificial heart muscle. **(b) Sensors for Functional Assessment**—Functional properties of 3D artificial heart muscle are recorded using sensors for twitch force, electrical properties, and calcium transients. The functional measurements are done independently using different sensors arrays for each measurement. In the idealized bioreactor system, functional properties of 3D artificial heart muscle can be measured using a single set of sensor arrays. In addition, the functional assessment can take place in real time and can make use of noninvasive sensor technology.

space is rapidly progressing to the point where advanced bioreactors are becoming a reality and fabrication of our idealized bioreactor is not far away.

The objective of the idealized bioreactor system is to provide physiological conditioning for 3D artificial heart muscle in order to support growth and maturation. The functional properties of artificial heart muscle from any research laboratory are lower than that of mammalian heart muscle. It is hypothesized that bioreactor conditioning will bridge the gap between the functional performance of artificial and mammalian heart muscle. Based on this hypothesis, our idealized bioreactor system has capabilities to deliver controlled electrical stimulation, uniaxial mechanical stretch, continuous media perfusion, and regulated growth factor stimulation (Figure 6.4a). These conditions accurately represent the culture

environment of the heart during normal mammalian function. The last of the four, growth factor stimulation, does not require device fabrication and therefore may not be considered a bioreactor; however, it is important in driving heart muscle phenotype.

Systems for individual stimuli are first shown independently for illustrative purposes and then combined into a single bioreactor. One can easily appreciate the degree of complexity associated with the idealized bioreactor system. This system is designed to condition artificial heart muscle using uniaxial stretch, electrical stimulation, continuous media perfusion, and chemical conditioning. As can be seen by the schematic, this system is extremely complicated, delivery of multiple signals is difficult, and device prototyping is challenging, to say the least.

Temporal and spatial variations in stimuli are needed. As an example, during the early stages of artificial heart muscle development, chemical conditioning needs to stimulate extracellular matrix production in order to support tissue growth and development. Therefore, the composition of growth factors would be optimized to support ECM production and include compounds like ascorbic acid and TGF-β. This needs to be followed by stretch protocols to support alignment of the newly formed ECM, and the intensity of the stretch protocol needs to be increased with the increase in ECM production. The rate of ECM production needs to be monitored and used as an input to adjust the stretch protocol. This is true for electrical stimulation and perfusion, and continuous exchange of information needs to take place between all four stimulation protocols.

The second part of our idealized bioreactor system consists of real time functional assessment of 3D artificial heart muscle (Figure 6.4b). The functional performance of artificial heart muscle can be evaluated by measuring the contractile properties, electrical properties, and calcium transients. In order to measure contractile properties, force transducers need to be attached to 3D artificial heart muscle, and in order to measure electrical properties, EKG catheters need to be engineered into the system. Calcium transients are measured by voltage-sensitive dyes, with changes in dye intensity correlating to changes in intracellular calcium concentrations. Measurement of any one of these functional properties is a challenging task; needless to say, the task of measuring all three functional performance metrics is a formidable challenge.

The final element of our idealized bioreactor system is communication between the two major systems—bioreactors and sensors. The sensors need to monitor functional performance of 3D artificial heart muscle using real time noninvasive monitoring technology; this data then serves as feedback to control stimulation protocols. As one example, embedded sensors will measure changes in calcium conduction velocity and can correlate this to intercellular connectivity. If a decrease in calcium conduction velocity is measured, it can be due to a decrease in intracellular connectivity. This data can then be used as an input for the bioreactors, which can modulate the stimulation protocol to support increased intracellular connectivity. One way of doing this process is by increasing the intensity of the stretch protocol to support intercellular alignment, connectivity, and as a result, support an increase in calcium conduction velocity.

6.6 BIOREACTORS AND TISSUE ENGINEERING

Bioreactor technology is critical for tissue engineering (Figure 6.5) and has been used to support every stage of the tissue fabrication process. The two are interrelated, and tissue fabrication is dependent on bioreactor technology to the extent that it is difficult to fabricate 3D artificial tissue in the absence of bioreactors. The significance of bioreactor technology in the tissue fabrication process cannot be underestimated, and the interdependent relationship has to be at the forefront of technological innovation in tissue engineering. Bioreactors have allowed the field of tissue engineering to progress by leaps and bounds, and continued progress in the field will depend on advancements in bioreactor technology.

Tissue fabrication can, and often does, take place in the absence of any bioreactors. This has been the case during the early years in the development of the field, when bioreactor technology was not as prevalent. However, significant improvements in process efficiency can occur through the implementation of bioreactor technology, and this improvement can in turn, lead to the fabrication of artificial tissue that is closer in form and function to mammalian tissue.

In order to better understand the relationship between bioreactor technology and tissue engineering, let us revisit the process of tissue fabrication. The first step required in tissue fabrication requires isolation, purification, and expansion of cells. This is followed by scaffold fabrication and cellularization of the scaffold

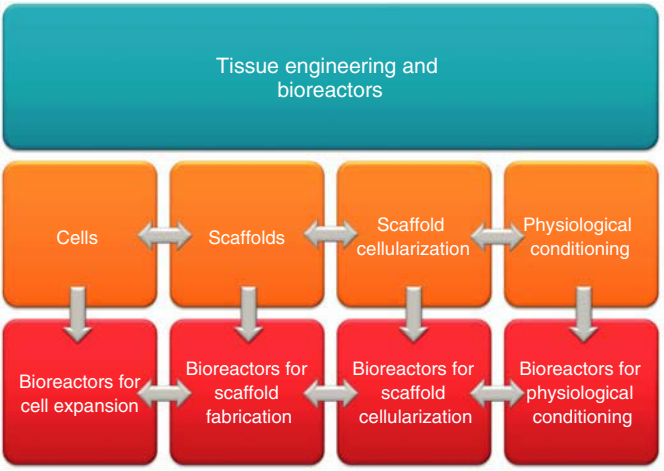

Figure 6.5 Integration of Bioreactor Technology with Tissue Engineering—This figure shows the close integration of bioreactor technology with tissue engineering and tissue fabrication. The figure illustrates core technologies required for tissue fabrication, including cells, scaffolds, scaffold cellularization, and physiological conditioning. Bioreactors can then be used to support core technologies necessary for tissue fabrication; this includes bioreactors for cell culture and expansion, bioreactors for scaffold fabrication, bioreactors for scaffold cellularization, and bioreactors for physiological conditioning (stretch, electrical stimulation, and continuous media perfusion).

with isolated cells. At this stage of the process, combination of cells with scaffolds results in the formation of functional 3D tissue. Artificial tissue at this early stage of development can be viewed as generation-one tissue, as it has some properties of mammalian tissue, but not all. The next step in the process is to support development and maturation of artificial tissue so that it is similar in form and function to mammalian tissue. One strategy to achieve this objective is by conditioning 3D artificial tissue using bioreactors to provide controlled stimulation for mechanical stretch, electrical stimulation, and continuous media flow.

Now that we have revisited the process of tissue fabrication, let us look at how bioreactors can be used at each step of the tissue fabrication process. During cell isolation and expansion, automated cell culture systems have been developed that have the capability of performing all related tasks using advanced robotics and control systems, thereby reducing the risk of contamination. Similarly, bioreactors are very often used for scaffold fabrication, and the process of electrospinning has gained popularity. Electrospinning is a tightly regulated process that supports fabrication of microfibers ranging in diameter from nanometers to micrometers (Figure 6.6).

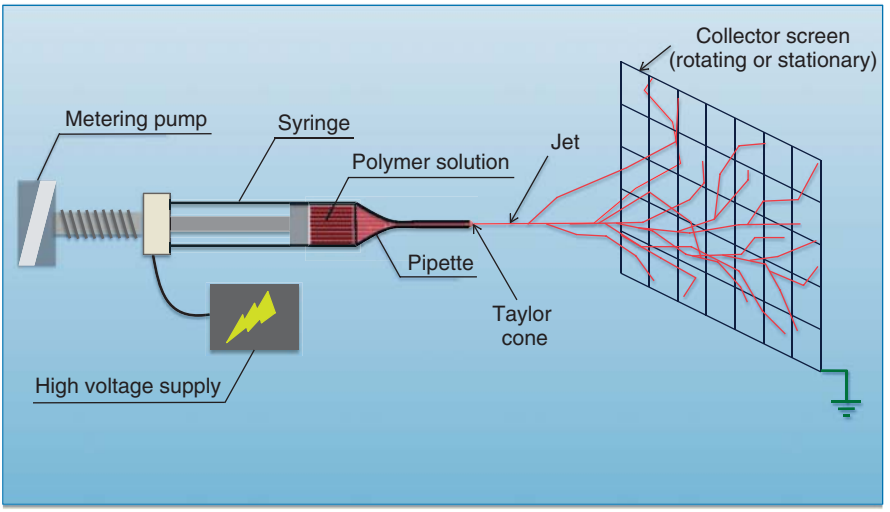

Figure 6.6 Electrospinning for Scaffold Fabrication—The process begins by solubilizing a polymer in a solvent and placing the polymer solution in a syringe. The polymer solution is held at the tip of the syringe by surface tension. One electrode is placed in the polymer solution and a second electrode is placed in the collection device. A high voltage power supply is used to propel the polymer solution out of the syringe by overcoming the surface tension that holds it in place. Gradually increasing the voltage results in the formation of a Taylor cone, which refers to the attachment of the polymer solution to the tip of the syringe in the shape of a cone. As the voltage is increased further, the fluid is ejected from the Taylor cone toward the collection device. As the polymer travels toward the collection device, the solvent evaporates, resulting in the formation of fibers. The fibers are collected in a collection device that can be configured to support the formation of 3D scaffolds.

Scaffold cellularization has also been supported by bioreactors using perfusion systems and other mechanisms for cell delivery within 3D scaffolds. This in turn results in increased cell retention and uniformity of cells distributed throughout the scaffold. Once 3D artificial tissue has been fabricated, bioreactors are used to deliver controlled stimulation to support the growth, development, and maturation of 3D artificial tissue. Bioreactors are used for mechanical stretch, electrical stimulation, and for continuous media perfusion.

6.7 BIOREACTORS FOR MAMMALIAN CELL CULTURE

Mammalian cell culture is at the heart of tissue engineering. Isolation, culture, and expansion of mammalian cells are critical prerequisites for tissue fabrication. Cell culture is one of the first techniques learned by new entrants to the field, including graduate and undergraduate students, and there are hundreds of cell culture facilities at large public universities. Mammalian cell culture is conducted using manual techniques, and for the most part, these manual techniques have been extremely successful. Traditional cell culture involves attachment and proliferation of adherent mammalian cells on tissue culture plates or flasks, with cell culture media being replaced every 1–2 days. Once cells reach confluency, trypsin is used to detach the cells from the culture surface; these cells are then replated at a lower density on multiple tissue culture plates. All of the techniques associated with mammalian cell culture have been conducted manually with a very high degree of success. Cell culture techniques are considered standard and routine in most tissue engineering research laboratories.

Based on the relative significance of mammalian cell culture in tissue engineering, it comes as no surprise that automated bioreactors for cell culture have been developed and are now commercially available. Automated cell culture bioreactors are designed to undertake all functions of mammalian cell culture using robotic technology. This involves culture and maintenance of cells on 2D monolayer surfaces, frequent media changes, enzymatic treatment to detach cells from the adherent surface, and subplating onto different monolayer surfaces. Automated mammalian cell culture systems are very sophisticated and require advanced engineering, control technology, and robotic systems for functionality. They offer clear advantages over manual cell culture by reducing overhead costs, personnel requirements, and the risk of contamination. However, these systems are expensive and therefore have not found wide acceptance in academic research laboratories.

An example of a bioreactor system for automated mammalian cell culture is the commercially available system, Cellerity™ from Tecan Group Ltd. The information presented here is based on publicly available information from the company website and from posters at conferences and other publications. The author does not endorse this product in any way, does not have any financial interest in the company, and does not have inside information about detailed engineering design for the system.

Let us begin by looking at some of the major components of the Cellerity system. Cellerity can be viewed as a cell culture laboratory condensed into a single unit with

advanced robotic and control systems. Atmospheric air enters the workspace and is filtered through a HEPA filter for sterilization, similar to the process of using a laminar flow hood. Cell culture plates are maintained in a CO_2 regulated incubator, which has robotic arms to transfer tissue culture plates from one location to another, depending on functional requirements. The robotic arms require advanced control systems and are designed to replicate physical movement of tissue culture plates that would otherwise be conducted by a human operator. Fluid handling requires several components, including refrigerators for bulk storage of media, robotic nozzles for fluid delivery, and vessels for waste collection. Cellerity also has automated cell counters and shakers. All processes are controlled using an advanced software program that has the capacity to control the frequency and volume of media changes, trypsinization, and subpassaging protocols, including regulation of plating density.

Tissue culture plates used for manual mammalian cell culture are not compatible with Cellerity and other bioreactors. A new generation of cell and tissue culture plates have been fabricated to support automated cell culture and are now commercially available. An example of such plates is roboflasks. These culture vessels have a large culture surface and have a lid, which vents to the atmosphere to promote gaseous exchanges and serves as an entry point for fluid exchange nozzles (required for media changes). During normal mammalian cell culture, roboflasks are positioned on their sides; however, they are used in an upright position during fluid exchange. There is also a venting port, which provides a sterile barrier to support gaseous exchange, built into the roboflasks. Fluid handling is done by robotic nozzles that pierce the lids on the surface of roboflasks and serve as an entry point for media aspiration and/or delivery.

Let us compare Cellerity with manual cell culture for maintaining cells on a 2D monolayer culture surface. We can assume that cells have been plated on the surface of tissue culture plates and are being maintained in a cell culture incubator, either as a part of Cellerity, or a stand-alone system in a traditional cell culture laboratory. In our example, we will compare the protocol for a single media change using Cellerity with the protocol for a single media change using manual methods.

Let us begin this discussion by providing a protocol for maintaining cells in culture the traditional way: using an operator based system. In this case, the operator will remove tissue culture plates from the incubator and transfer them to a laminar flow hood that has been sterilized with ethanol. Spent media will then be aspirated from the culture plates using a vacuum system, and fresh media, which has been pre-heated to 37°C, will be added to the culture plates. Once all culture plates have been processed, they will be transferred from the laminar flow hood to the cell culture incubator. Depending on the cell type, this process will be repeated every 1–2 days for an average of 7–10 days.

Now let us look at the same protocol using Cellerity. Roboflasks will be maintained in an incubator chamber and transferred to the culture surface using robotic arms. The culture plates will be secured in the housing, and spent media will be aspirated using robotic nozzles while fresh media, which has been perfused through a heating coil, will be delivered to the plates, again using robotic nozzles.

The roboflasks will then be returned to the incubator chamber using robotic arms. The frequency of media changes, along with the volume, will be programmed using software.

Let us compare the relative advantages and disadvantages of the manual versus automated methods for media changes. The most significant advantage is obvious—the automated process does not require direct human control and therefore is more cost effective (excluding capital expenditure) and less prone to contamination. The biggest disadvantage of the automated system is indeed the initial capital investment, which is lower for a traditional cell culture experimental setup than automated bioreactors. If large-scale studies are being conducted, an automated system will be more cost-effective, and the extra work of having to train multiple personnel to perform traditional cell culture techniques can be significantly reduced or eliminated altogether.

We will end our discussion on bioreactors for mammalian cell culture by answering one question—*can these systems provide a valuable tool for tissue engineering?* From a tissue engineering standpoint, the objective is often, if not always, to expand the number of cells to obtain large cell numbers for tissue fabrication. The expansion of mammalian cells requires numerous tissue culture plates, often reaching in excess of one hundred. Handling such a large number of tissue culture plates manually can prove to be challenging and labor intensive, and due to the repetitive nature of media changes, this manual handling can increase the likelihood of contamination. Automated cell culture systems provide a valuable tool for such large-scale studies by eliminating or reducing operator-dependent errors and therefore lead to process efficiencies and cost reduction.

6.8 BIOREACTORS FOR SCAFFOLD FABRICATION

In this section, we look at electrospinning as one example of bioreactors that have been used for scaffold fabrication. Electrospinning is a method fabricating individual fibers of a polymer that can be combined in different configurations to promote 3-dimensional scaffold fabrication (7–12). Scaffolds have been fabricated using electrospinning with a wide array of polymers, including polylactic acid, poly(glycolide), polyurethane, polystyrene, collagen, fibrinogen, gelatin, hyaluronic acid, and chitosan. In the electrospinning process, the polymer is first solubilized, and polymer solution is introduced into a syringe. The polymer solution is held in position at the end of the syringe tip by surface tension. A high voltage is used to deliver an electric field to the polymer solution; this step results in the polymer being charged from the syringe tip. The electric field acts in the opposite direction of the surface tension, and when it overcomes surface tension, a charged jet of polymer solution is extruded from the syringe. Ejection of the polymer solution from the syringe results in formation of a Taylor cone—attachment of the polymer solution to the tip of the syringe in the shape of a cone. As the voltage is increased further, fluid is ejected from the Taylor cone and travels toward the collection device. As the fluid travels, the solvent

evaporates, resulting in the formation of thin fibers. These fibers are collected in a collection device that can be configured to support the formation of 3D scaffolds (Figure 6.6).

What are the important processing parameters affecting the electrospinning process? We now look at ways to control the electrospinning process which determine properties of the scaffold. The most important variables that regulate the electrospinning process are: 1) viscosity and concentration of polymer solution, 2) molecular weight of polymer solution, 3) conductivity of polymer solution, 4) surface tension at tip of syringe, 5) voltage of the electric field, 6) distance between syringe tip and collector, and 7) design and configuration of the collector. Let us look at these seven variables further.

Viscosity and Concentration of the Polymer Solution—Viscosity and concentration of the polymer solution are related, as increasing polymer concentration leads to an increase in solution viscosity. Increasing viscosity of the polymer solution translates to having more polymer per unit volume of solution and less solvent. As the polymer jet travels from the syringe tip toward the collector, evaporation of the solvent takes place; since there is less solvent at the start of the electrospinning process, evaporation results in excessive drying of the polymer fiber, which adversely alters 3D scaffold fabrication. Now let us look at the reverse argument. If we begin the electrospinning process with a low viscosity polymer solution, this translates to having less polymer per unit volume of solution and more solvent. As the jet travels from the syringe tip to the collector, the solvent is not able to completely evaporate due to the presence of a greater solvent per volume of polymer solution. When the jet of polymer solution reaches the collector, it is wet, and as before, this wetness adversely affects 3D scaffold fabrication.

Molecular Weight of Polymer Solution—This relationship can be explained based on the relationship between the molecular weight of the polymer and the viscosity of the polymer solution; the higher the molecular weight of the polymer, the higher the viscosity of the solution with all other variables remaining constant. There is a correlation between the molecular weight and fiber diameter; increasing molecular weight of the polymer leads to an increase in fiber diameter, while decreasing molecular weight of the polymer leads to a decrease in fiber diameter or bead formation. Bead formation refers to the inability of the electrospinning process to produce polymer fibers; rather than formation of fibers, beads are formed. This bead formation is a negative outcome that adversely affects 3D scaffold formation.

Conductivity of Polymer Solution—Conductivity of the polymer solution affects the ability of any given applied electric field to eject the polymer solution from the syringe tip. Polymers with low conductivity are ejected from the syringe tip with a lower force compared to polymers with high conductivity. While there are some discrepancies in the relationship between polymer conductivity and fiber diameter, within a critical range of polymer conductivities, it has been found that there is a decrease in fiber diameter with increasing conductivity of the polymer solution. Within the critical range of polymer conductivity, increasing polymer conductivity leads to an increase in ejection velocity, which in turn increases solvent evaporation

leading to a decrease in fiber diameter. If a polymer solution has low conductivity outside of the critical range when an electric field is applied, the force of ejection is low, and this results in inadequate elongation of the polymer fiber; this elongation in turn leads to a reduction in fiber stability, uniformity, and can prevent fiber formation altogether. On the other hand, if the polymer solution has conductivity higher than the critical range, there is a high force of ejection when the electric field is applied; this high force of ejection leads to unstable fibers and can prevent fiber formation altogether.

Surface Tension at Syringe Tip—Surface tension serves to hold the polymer in place prior to application of an electric field. Once the electric field is applied, the surface tension has to be overcome and the polymer is ejected from the collector. If the surface tension of the polymer solution increases with all other variables remaining the same, the polymer solution will "hold on" to the syringe tip with a greater force; this means that application of a given electric field will not be sufficient to eject the polymer solution from the syringe tip. If the polymer solution is retained at the polymer tip or if less polymer is ejected toward the collection vessel, it will lead to instability in fiber formation and can also lead to the formation of beads. The higher the surface tension of the polymer, the greater the magnitude of the problem.

Effect of Applied Electric Field—The properties of the electric field play an important role in the electrospinning process and serve to overcome surface tension at the syringe tip and eject the polymer solution toward the collection device. An increase in applied voltage will result in an increase in the amount of polymer being ejected from the syringe tip; this ejection will in turn result in an increase in fiber diameter. While an increase in the electric field results in an increase in fiber diameter, this relationship is dependent on the conductivity of the polymer. In the case of highly conductive polymers, the reverse relationship applies: an increase in applied electric field results in a decrease in fiber diameter. In the case of highly conductive polymers, the electric field serves to destabilize the polymer due to an increase in electrostatic repulsive forces within the polymer solution; this in turn can result in a decrease in fiber diameter or disrupt fiber formation altogether.

Distance between Tip and Collector—The distance the polymer jet travels is determined by the placement of the collector relative to the syringe. If this distance is too small or too large, it will hamper scaffold formation and can result in wet fiber or bead formation. If the collector is placed too close to the syringe, there is a reduction in the time available for the polymer jet to travel to the collector. This in turn results in reduced solvent evaporation resulting in the formation of wet fibers. If the collector is placed too far from the syringe, excessive solvent evaporation takes place and can lead to excessive drying, which will result in bead formation.

Design and Configuration of Collector—The orientation of the fibers is dictated by the design configurations of the collector. Planar scaffolds can be fabricated with planar collection vessels, while cylindrical scaffolds can be fabricated with rotating collection vessels.

6.9 BIOREACTORS FOR SCAFFOLD CELLULARIZATION

Fabrication of 3D artificial tissue requires cellularization of scaffolds. In the simplest embodiment, cells are injected into complex 3D scaffolds using a syringe as the delivery vehicle, with multiple injections being required for cellularization. Success of the cellularization strategy is measured based on cell viability, cell retention, cellular alignment, uniform distribution of cells, and functional coupling between adjacent cells and with extracellular matrix. As can be envisioned, direct cell injection strategies are not always the most efficient at achieving the stated objectives due to many problems, including low cell retention within the scaffold. Bioreactors have been developed to aid the cellularization process, and in this section we will discuss six cellularization methods: 1) direct cell injection, 2) cell entrapment using hydrogels, 3) perfusion seeding, 4) surface acoustic waves, 5) centrifugal force, and 6) magnetic nanoparticles. These cellularization strategies are presented in Figure 6.7.

Direct Cell Injection—Direct cell injection is the simplest strategy for scaffold cellularization and is the oldest and most widely used method (18) (Figure 6.7a). In this method, primary cells are suspended in cell culture media and transferred to a syringe or other delivery vehicle. These cells are then directly injected on the surface of the scaffold or injected within the fibers of the 3D scaffold. In most cases, multiple injections on several sites on the scaffold are required for complete cellularization. The scaffold is left undisturbed for 1–2 hours to allow cells to populate the scaffold and then is transferred to an incubator for culture. The advantages of direct cell transplantation are that it is simple, does not require expensive instrumentation or training for personnel, and can be completed within a short time period. There are, however, some limitations with these methods. Delivery of the cells through a needle or syringe can lead to cell damage and loss of viability, particularly if the orifice size is small. Direct injection of cells within the scaffold does not always result in uniform cell distribution, as the process does not involve any degree of precision. Cell retention is low, and the cells have a tendency to be washed out either immediately upon cellularization or after the first media change. Although there are known limitations with direct cell injection methods, these methods continue to be preferred for scaffold cellularization to support tissue fabrication due to the simplicity of the method and ease of implementation.

Cell Entrapment using Hydrogels—One of the major limitations with direct cell injection methods is low cell retention, as the injected cells have a tendency to leak out of the scaffold. A second category of cellularization strategies has been developed to address this limitation and is known as cell entrapment using hydrogels. The idea is to trap the cells within a biodegradable hydrogel during cellularization, thereby reducing the likelihood that cells will leak out of the scaffold (19). The hydrogel is designed to secure the cells in place within the 3D scaffold and is designed with tunable degradable kinetics. During the initial stages of scaffold cellularization, the biodegradable hydrogel serves to hold the cells within the scaffold. As cells populate and cellularize the 3D scaffold to form functional tissue, the hydrogel degrades over time using hydrolysis, enzymatic treatment, or some other degradation method. By this time, cells generate their own extracellular matrix,

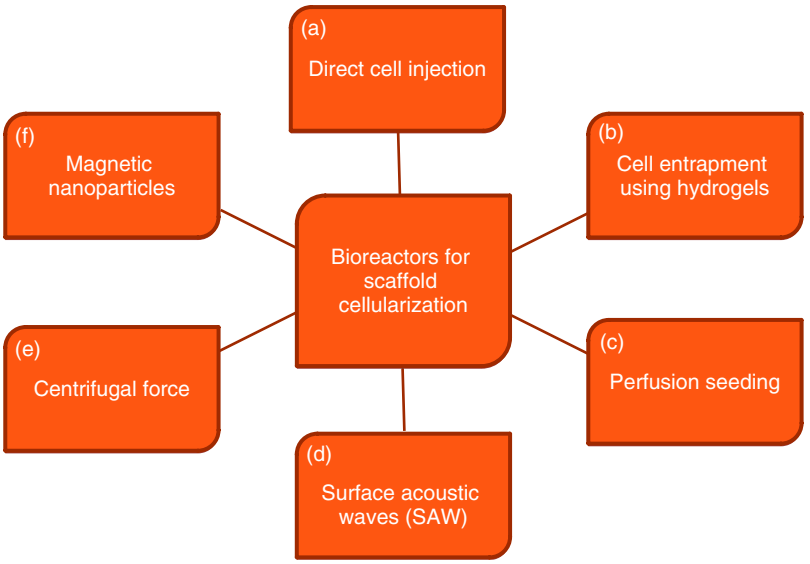

Figure 6.7 Bioreactors for Scaffold Cellularization—(a) Direct Cell Injection—Cells are suspended in culture media, placed in a syringe, and directly injected into the 3D scaffold. **(b) Cell Entrapment using Hydrogels**—Cells are suspended in a thrombin solution, placed in a syringe, and directly injected into the 3D scaffold, which has been presoaked in a fibrinogen solution. The addition of thrombin promotes polymerization of the fibrinogen and results in the formation of fibrin gel. **(c) Perfusion Seeding**—Porous channels are fabricated within 3D scaffolds. Cells are suspended in cell culture media and perfused through the scaffold using a peristaltic pump. Perfusion of the cell suspension results in cells entering into the porous channels of the scaffold, which supports scaffold cellularization. **(d) Surface Acoustic Waves (SAW)**—Cells are suspended in culture media and are placed in a chamber. IDT electrodes are used to generate SAW, which guides movement of cells towards the 3D scaffold. **(e) Centrifugal Force**—3D scaffold is positioned within a conical tube. Cells are suspended in culture media and added to the conical tube. The conical tube is placed in a centrifuge and subjected to a centrifugal force, which supports cellularization of the 3D scaffold. **(f) Magnetic Nanoparticles**—Magnetic nanoparticles are combined with a cell suspension and directly added to a scaffold. Magnetic force is used to guide and position cells within the 3D scaffold.

which is used to retain cells and support formation of 3D tissue. The biodegradable hydrogel, therefore, acts as a temporary support matrix.

One example of cell entrapment technology is fibrin being used as the biodegradable hydrogel for cell entrapment (19) (Figure 6.7b). Fibrin is a commonly used hydrogel in tissue engineering. It is formed by the polymerization of fibrinogen by thrombin. In this example, cells are suspended in a solution that contains thrombin while the 3D scaffold is soaked in fibrinogen. The cell suspension is added to the scaffold using a syringe. As the thrombin comes in contact with fibrinogen, fibrin gel forms and traps cells in place.

The two methods of scaffold cellularization that have been described so far, direct cell transplantation and cell entrapment using hydrogels, are not based on bioreactor technology. These two methods are included in our discussion for the sake of completion and to compare these methods with methods that rely on bioreactor technology, as we will see for the remainder of this discussion.

Perfusion Seeding—Perfusion bioreactors have been used to support scaffold cellularization, and here we look at one specific example from the literature (20). In this case, porous scaffolds were designed with pore sizes in the range of 75–100 μm and flow channels with a diameter of 250 μm. The flow channels were designed to serve as the entry point for cells within the 3D scaffold, and the pores were designed as attachment points for cells. For scaffold cellularization, cells were suspended in culture media and perfused through the flow channels; the direction of the flow was changed at regular intervals. As the cell suspension was perfused through the flow channels, cells traversed from the flow channels to the pores within the 3D scaffold and were trapped within these pores. This method of perfusion seeding has been shown to result in uniform cell distribution with seeding efficiencies of up to 80%. Perfusion seeding is advantageous, as it uses fluid flow to support entry of cells within 3D scaffolds rather than passive seeding, which in effect, forces cells into the scaffold. This way, perfusion seeding increases seeding efficiency and regulates spatial distribution of cells based on flow channel geometry. The major limitations of perfusion seeding are the need for specialized perfusion apparatus, custom designed scaffolds, and specialized training for personnel, all of which lead to an increase in cost and time.

Surface Acoustic Wave Technology—Surface acoustic waves (SAWs) are acoustic waves that are generated on the surface of a piezoelectric substrate and travel along the surface of the material. Their amplitude typically decays exponentially with depth into the material (21). SAWs are frequently used in electronic circuits as filters, oscillators, and transformers. SAWs are generated by interdigital transducer (IDT) electrodes and travel from an input transducer toward an output transducer (21) (Figure 6.7d). In this case, IDT electrodes were engineered onto a piezoelectric substrate and used to generate SAWs by application of a radio frequency. In this example, the SAW travels from the input transducer to the output transducer. A 3D scaffold is positioned in the path of the SAW, and a droplet of fluorescent beads is positioned in the path of the SAW in close proximity to the scaffold. As the SAW travels from the input to the output transducer, the fluorescent beads travel toward the scaffold and populate the 3D scaffold. This process has shown to be very rapid, and cellularization can be accomplished within seconds, compared to hours for other cellularization strategies like direct cell transplantation. SAW bioreactors for scaffold cellularization is a fairly new technology and is limited to a few specialized laboratories with the required engineering capabilities. However, SAW technology is promising and has the potential to be used in beneficial ways for the tissue fabrication process.

Centrifugal Force—Centrifugal force has been used as a strategy for cellularization of scaffolds. In one example, scaffolds were positioned within a conical tube and then submerged in cell suspension (22). The conical tube with the scaffold

and cell suspension was placed in a centrifuge and subjected to centrifugal force (Figure 6.7e). The centrifugal force resulted in transfer of the cells to the interior of the scaffold. After cellularization, the scaffold was removed from the conical tube and cultured within a cell culture incubator. This strategy was shown to significantly improve cellularization efficiency when compared to direct cell transplantation.

Magnetic Nanoparticles—Another technique that has been used for scaffold cellularization is known as magnetic force-based tissue engineering, or Mag-TE (23). Mag-TE methods make use of magnetite cationic liposomes (MCLs), which are nanoparticles with a diameter of approximately 150 nm that contain magnetite nanoparticles (Fe_3O_4). MCLs interact with cells, and the magnetite nanoparticles result in cells being charged; this property of cells is then used as a tool for scaffold cellularization (23) (Figure 6.7f). In one example, MCLs were first mixed with a cell population. Magnetically labeled cells were then added to the surface of a 3D scaffold that was placed on the surface of a magnet. The cells travelled through the scaffold starting on the surface and gradually progressing inward; this movement was controlled by the magnetic field created by the underlying magnet. This process resulted in uniform cell distribution throughout the 3D scaffold. Mag-TE is a novel method to support scaffold cellularization and can have a significant impact on the field of tissue engineering. However, high levels of expertise are required for the fabrication of MCLs, which are not commonly used in many labs. Investment is also required for personnel training, adding to the time and cost of implementation of Mag-TE.

6.10 PERFUSION SYSTEMS

Need for Perfusion Systems—In the human body, the circulatory system acts as a distribution network for the delivery of nutrients to cells and tissues while at the same time removing waste products. All cells in the human body are within a couple hundred microns from a capillary; this characteristic is essential to support cell viability. During 2D monolayer culture, cells are cultured on the surface of a tissue culture plate and cell culture media is replaced every second or third day, depending on cell type. This strategy is also used to support the culture of 3D artificial tissue. While adequate to support the culture of cells and tissue, these culture conditions do not replicate *in vivo* physiological flow conditions. For example, media changes every second or third day does not mimic blood flow conditions and is not adequate to support cell viability during culture of 3D artificial tissue. This alludes to the fact that bioreactor systems, which are capable of delivering continuous fluid flow to support the metabolic activity of cells and artificial tissue during controlled *in vitro* culture, are needed. The need for perfusion systems is greater for 3D artificial tissue than for cells during 2D monolayer culture due to an increase in metabolic activity resulting from an increase in tissue mass of 3D artificial tissue (24–36).

Control of Processing Variables in Perfusion Systems—Perfusion systems can be designed to operate inside of a cell culture incubator or completely independent of a cell culture incubator, with the former strategy being more common.

The advantage of designing a perfusion system to function within cell culture incubators is the ability to regulate processing variables like temperature, pH, and CO_2/O_2 levels using feedback control loops within the cell culture incubator. There is, however, a significant limitation with the use of cell culture incubators, as these devices have fixed configurations and do not allow significant user control over processing variables. Design and fabrication of such perfusion systems that rely on the use of cell culture incubators to regulate processing variables is simple. However, if user control over processing variables is required, the perfusion system has to be fabricated with onboard sensors and feedback loops for regulation of temperature, pH, and CO_2/O_2 levels. In this case, perfusion systems are more complex, but they have the advantage of user control over processing variables, which can be changed based on the specific tissue fabrication application. While this process is lengthy and expensive, in the long run, it delivers the level of control that is necessary for tissue engineering applications. The main advantage of embedding sensors within bioreactors is the degree of flexibility in controlling processing variables and the ability to fine-tune the specific processing parameters based on tissue-specific requirements.

There are many processing variables that need to be regulated during perfusion culture of 3D artificial tissue, the most important of which include temperature, pH and gaseous composition. If perfusion bioreactors are being designed to function within a cell culture incubator, then these processing variables are accurately regulated within the incubator. If the perfusion bioreactors are being designed as stand-alone systems, then control of temperature, pH, and gaseous composition is performed by the bioreactor. Temperature can be controlled in one of two ways: either by the use of water-jacketed reservoirs or temperature-regulated metallic plates (Figure 6.8a).

In the first case, water-jacketed reservoirs are fabricated around the chamber or reservoir to be regulated; hot water is perfused through these reservoirs using a thermocirculator, which is a piece of equipment with a heating element and pump. Heat exchange from the hot water to the chamber or reservoir results in a temperature increase within the culture chamber/reservoir. The second approach involves the use of a metallic plate, attached to a heating element with feedback control, to regulate temperature of the culture environment. The feedback control loops serve to regulate the temperature at a preset value. Both methods are fairly accurate for temperature regulation and have been used extensively.

Regulation of pH is achieved in one of two ways (Figure 6.8b). The first method involves the use of CO_2 to regulate pH of the culture media, just as is the case in a cell culture incubator. CO_2 is injected within the culture chamber or media reservoir and acts to lower the pH to physiological values of 7.4. Once the pH reaches a user-defined preset value, say 7.3, CO_2 flow to the media is stopped. As a result, the pH of the cell culture media or culture environment begins to rise, and once the pH reaches a second user defined preset value, say 7.5, the flow of CO_2 is re-initiated; this serves to lower the pH of the culture media. Using this strategy, the pH of the cell culture media or culture chamber can be maintained with a narrow user-defined range, typically 7.3–7.5. The use of CO_2 for pH regulation

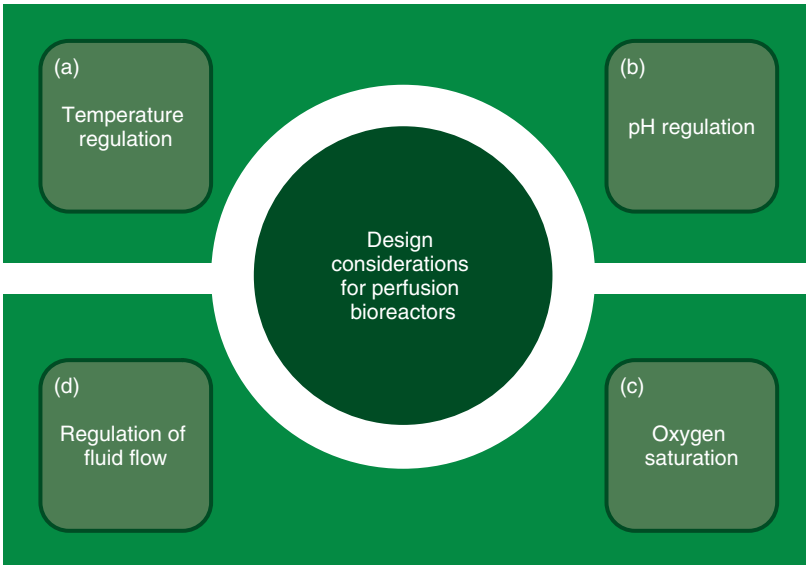

Figure 6.8 Design Considerations for Perfusion Bioreactors—(a) Temperature Regulation—Water-jacketed reservoirs are used for temperature regulation in cell culture reservoirs and incubation chambers. Alternatively, a heating element with a thermostat for temperature regulation can be used. **(b) pH Regulation**—CO_2 injection with feedback control can be used to regulate the pH of cell culture media at physiological levels for long-term studies. For short-term studies, pH regulation can be achieved using buffers. **(c) Oxygen Saturation**—Oxygen saturation can be regulated by direct bubbling of oxygen to the cell culture media. Alternatively, a cell culture incubator with oxygen regulation capabilities can be used with perfusion bioreactors. **(d) Regulation of Fluid Flow**—A single peristaltic pump can be used for media delivery and aspiration, or a dual pump configuration can be used, with a single peristaltic pump being used for media delivery and a second one being used for media aspiration.

is complex and requires CO_2 sensors, control valves, and feedback control algorithms. CO_2 regulation as a strategy to maintain pH is used in perfusion systems only when the specific application requires long-term culture, usually ranging from weeks to months. For short-term culture applications, ranging from hours to days, there are many commercially available buffers that are excellent for pH regulation, including 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and sodium bicarbonate.

Regulation of a gaseous environment requires control of CO_2 level for pH control (as we have seen before) and oxygen saturation (Figure 6.8c). Oxygen saturation of the cell culture media is an experimental variable and varies from application to application. In some cases, hypoxic conditions are required and oxygen saturation has to be adjusted to less than 1%, and in other cases, higher oxygen saturation is needed, sometimes in excess of 50%. The atmospheric oxygen concentration

is 21% (and 78% nitrogen). If any tissue engineering application requires oxygen concentration in excess of 21%, oxygen is injected in the culture chamber or bubbled in the cell culture media; conversely, if any given application requires lower oxygen saturation, nitrogen is injected into the culture chamber and acts to displace oxygen from the culture environment. The exact oxygen concentration needs to be measured with sensors and embedded feedback control systems. This is not a trivial task and requires advanced sensor and control technology.

Components of Perfusion Systems—Many components are required to assemble a perfusion system, one of which is the housing for 3D artificial tissue; several design variables need to be taken into consideration when fabricating housing for 3D tissue. The most important variable is the transfer of 3D artificial tissue from the culture vessel to the perfusion chamber; *will the entire culture plate will be transferred or just the 3D artificial tissue?* The former strategy is preferred, as it does not require physical handling of artificial tissue, which can lead to damage and loss of function. Another important decision is the time at which perfusion is initiated. When artificial tissue is cultured in the presence of continuous media flow, perfusion is initiated a few days after tissue fabrication. This is to allow time for the cells to attach to the scaffold and support 3D tissue formation; if perfusion is started too early, cells can be washed away, leading to tissue damage.

Pulsatile pumps are frequently used to replicate the pulsatile behavior of blood flow; programmable pumps are used to deliver advanced flow regimes like sinusoidal waves (Figure 6.8d). At times, a single pump is sufficient to support media delivery and aspiration to the culture plates, while for some applications, two pumps are required: one for media aspiration and a second pump for media delivery. If a single pump is used, the rate of fluid delivery and aspiration needs to be balanced to prevent media overflow; this is sometimes a difficult task to achieve, and therefore, dual pump models are frequently used.

An Example of a Perfusion System—The system consists of a custom biochamber designed to accommodate 10 tissue culture plates with each plate stacked vertically on an independent stage (16). Cell culture media was maintained in a temperature-regulated reservoir and was delivered to the tissue culture plates via a peristaltic pump. Two specialized manifolds were precision-fabricated to allow simultaneous placement of the fluid flow ports to each chamber. A single peristaltic pump drives the inflow, drawing from a water-jacketed, temperature-controlled reservoir. Media oxygenation was accomplished by a membrane oxygenator. Media was aspirated from each plate utilizing custom-designed manifolds attached to a vacuum line. The aspirated media was recycled to the media reservoir. High relative humidity was maintained in the biochamber by delivering moist air from a humidification chamber. The inflow and outflow fluid manifolds were configured with inline measurement of O_2, pH, and temperature. Threaded Luer-Lock ports at the top of the chamber, in conjunction with single direction check valves, allowed for gas inflow and outflow without the possibility of reverse flow contamination.

6.11 BIOREACTORS FOR STRETCH

Introduction—Stretch is an important modulator of physiological function. All mammalian cells have biological machinery to sense and respond to changes in the stretch environment. This is particularly important in the cardiovascular system where cells are constantly exposed to a variety of hemodynamic forces and must respond to these changes to maintain normal physiological function. Cells have biological force sensors, which respond to changes in the force environment, embedded within the cell membrane; these biological force sensors are known as stretch-activated channels (SACs) (37–48). In response to changes in the extracellular stretch environment, SACs undergo a conformational change leading to a cascade of intracellular signaling events. The intracellular signaling cascade leads to changes in the biological functions of the cell by inducing changes in the gene and protein expression pattern. In this manner, SACs are able to respond to the extracellular stress environment by eliciting an intracellular response.

In the next section, we will look at the effect of stretch on cells of the cardiovascular system, namely vascular smooth muscle cells, endothelial cells, and cardiac myocytes. We will then discuss bioreactors that have been developed to deliver controlled stretch of cells/tissue for the cardiovascular system. Let us begin our discussion by looking at the effect of stretch on vascular smooth muscle cells.

Effect of Stretch on Vascular Smooth Muscle Cells—All blood vessels are constantly subjected to fluid shear stresses in response to pulsatile blood flow, and vascular smooth muscle cells (SMCs) and endothelial cells (ECs) constantly remodel in response to these pulsatile fluid stresses. In response to stretch, SMCs are known to exhibit a host of phenotypic changes, including an increase in the rate of proliferation and expression of contractile proteins (49–58). In SMCs, zyxin is an important mechanosensitive protein that binds to the cytoskeleton proteins actin and vinculin to form a focal adhesion complex. In response to stretch, zyxin rapidly accumulates at the point at which stress is applied and promotes recruitment of α-actinin and vasodilator-stimulated phosphoprotein (VASP), which serve to stabilize intracellular actin fibers. Many of the downstream effects of stretch are mediated by RhoA, which is a GTPase protein that acts on rho-associated coiled-coil-containing *protein (*ROCK), and this signaling pathway leads to an increase in the expression of SMC contractile proteins.

Effect of Stretch on Endothelial Cells—Endothelial cells (ECs) line the luminal surface of blood vessels within the vascular system and are constantly exposed to the hemodynamic force resulting from pulsatile blood flow. ECs play an important role in regulating vascular tone and act to regulate the properties of SMCs. In effect, ECs are the first responders to changes in the physiological hemodynamic environment and therefore have an extensive biological system in place for sensing and transmitting mechanical forces. ECs have several ion channels located on the membrane surface that are known to respond to changes in fluid hemodynamics, some of which include transient receptor potential channels (TRPs), P2X4 purinoreceptors, potassium channels, and chlorine channels (59–68). In addition to ion channels, ECs also have other types of mechanosensors, including integrins,

platelet endothelial cell adhesion molecule-1 (PECAM-1), VE-cadherin, caveolae, G proteins, glycocalyx, and the endothelial cell cytoskeleton.

In response to stretch, these mechanosensors act to increase intracellular calcium contractions, which in turn results in an increase in the vasodilator, nitric oxide (NO); this increase in NO leads to vasodilation. Shear stress is also known to result in an increase in the expression of many proteins in ECs, including cytoskeletal and matrix proteins.

Effect of Stretch on Cardiac Myocytes—Just as blood vessels are constantly exposed to pulsatile fluid flow, the heart is also under constant hemodynamic stress, and cardiac cells, including fibroblasts and myocytes, respond to these changes by regulating intracellular molecular and cellular events (69–75). Under normal physiological conditions, the heart responds to changes in hemodynamic loads by increasing extracellular matrix components and by hypertrophic and hyperplasic growth of cardiac cells. In addition, pressure overload in the heart leads to an increase in the rate of proliferation of cardiac fibroblasts associated with an increase in expression of collagen type I. Changes in hemodynamic loading resulting from volume overload are also associated with cardiac myocyte hypertrophy in addition to increases in collagen deposition.

Stretch and Tissue Engineering—Our discussion until this point has served to demonstrate the role of mechanical stretch and/or pulsatile fluid flow on modulating the cellular and molecular properties of cardiovascular cells, including SMCs, ECs, and cardiac myocytes. This serves to illustrate the critical role of stretch in maintaining and modulating tissue function by regulating cellular and molecular properties. Moving on from this discussion, we need to ask one question - *how can this information be applied to tissue engineering?* Our objective in tissue engineering is to fabricate artificial tissue and develop *in vitro* culture conditions that mimic *in vivo* physiological conditions in order to support growth and maturation of 3D artificial tissue. As can be seen from our previous discussion, stretch can be used as a modulator of cell and tissue function. Therefore, applied to tissue engineering, the objective is to design and fabricate of custom bioreactors to deliver controlled stretch protocols to support growth and maturation of 3D artificial tissue. Let us look at one example of a bioreactor that has been developed to deliver controlled stretch to modulate the function of 3D artificial heart muscle.

Design Variables for Stretch—We begin by looking at some of the important design variables that need to be taken into consideration. Any given stretch protocol can be defined by percentage stretch, frequency, and duration of stretch. The percentage stretch refers to the displacement of the tissue construct in response to the stretch protocol; for example, if artificial tissue is planar and measures 20 mm × 20 mm, a 10% stretch would require a displacement of 2 mm for every stretch cycle. Frequency of stretch refers to the number of cycles per second and is measured in Hz—a frequency of 1 Hz refers to one cycle per second. Stretch duration refers to the time for which the stretch protocol is implemented, ranging from minutes to weeks. Uniaxial stretch refers to stretch protocols in one direction, while biaxial stretch refers to stretch in two directions (x-y plane). Stretch velocity refers to the time required to complete a single stretch cycle. For example, if a displacement of

2 mm is required and this process required 1 second to complete, the stretch velocity is 2 mm/s. Rest periods are also integrated within the stretch protocols, particularly when working with systems involving skeletal muscle, which has a high degree of fatigability.

Now that we have some indication of the variables that define stretch protocols, the next question to address is: *what stretch protocol should we begin with for any given 3D artificial tissue?* It is instinctive to use stretch protocols that are based on *in vivo* parameters for *in vitro* conditioning of artificial tissue. For example, if we have to develop a stretch protocol for artificial heart muscle, we will instinctively draw inspiration from stretch parameters in the heart and replicate these conditions *in vitro*. Often, this is not the best strategy, as 3D artificial tissue does not have the same architecture as mammalian tissue and cannot withstand harsh stretch protocols. When developing stretch protocols for 3D artificial tissue, the objective should always be to start with mild stretch protocols, validate tissue compatibility, and then gradually increase the intensity of the stimulation protocols.

An Example of a Stretch Bioreactor—We describe a stretch bioreactor designed to readily accommodate a range of self-organizing tissues, including skeletal, cardiac, and smooth muscle, tendon and ligament, and bone (14). Adjacent to each 35 mm plate is a movable post. One end of each tissue specimen is affixed to this post via a stainless steel wire that is shaped to allow it to pass under the cover of the 35 mm dish and attach to the stainless steel insect pins that affix the ends of each tissue specimen. The 35 mm plates do not require modification; the lids are simply tipped forward to accommodate the stainless steel wire. The culture plates are held stationary above a moving platform to which the movable posts are affixed below. Thus, during mechanical movement of the posts, the tissue specimen length is changed, but the base of the culture dish remains motionless. This arrangement minimizes sloshing of the culture media that would occur if the culture dish were moved cyclically or dimensionally deformed. The mechanical strain mechanism is comprised of a linear actuator that drives the moving platform beneath the culture plates. The linear actuator is a direct-drive ACME miniature power screw affixed to the output shaft of the gear-head step motor. Mechanical strain of the same amplitude is applied to all specimens simultaneously. In general, different mechanical stimulus protocols would be carried out on separate but identical bioreactors. Mechanical strain is controlled by a gear-head stepper motor driven by an open-loop stepper motor driver.

6.12 ELECTRICAL STIMULATION

Introduction—In this section, we will look at bioreactors for controlled electrical stimulation of 3D artificial tissue. The use of electrical stimulation as a treatment modality has been around for decades and has been used in numerous applications, ranging from cardiac pacemakers to vagus nerve stimulation for treatment of seizures during epilepsy. During normal mammalian function, changes in voltage are used as a trigger to modulate cell and tissue level function. These changes

in voltage are brought about by changes in ion concentration within the cellular and/or extracellular environment, which leads to conformational changes in voltage-sensitive channels. We begin this section with a discussion of specific applications of electrical stimulation as a treatment modality outside of the tissue engineering space.

Electrical Stimulation as a Treatment Modality—Cardiac pacemakers are used to synchronize cardiac contraction in cases of arrhythmias or tachycardia (76–79) (Figure 6.9a). Pacemakers consist of a control unit,which is designed to monitor activity of the native heart and deliver specific electrical impulses to the electrodes, and the actual electrodes, which are implanted in contact with the right atrium, ventricle, and heart muscle. The pacemaker is implanted under the collarbone, and

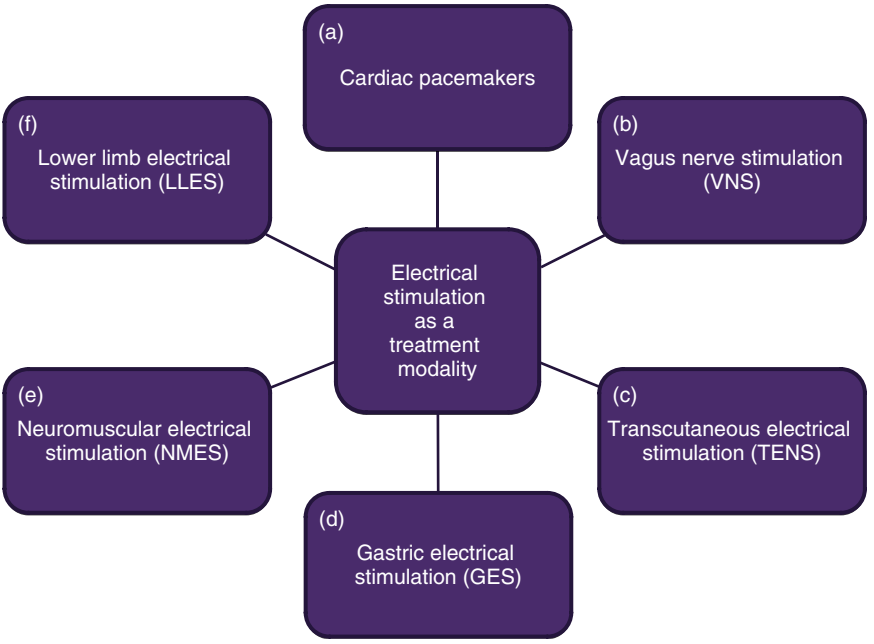

Figure 6.9 Electrical Stimulation as a Treatment Modality—(a) Cardiac Pacemakers—Cardiac pacemakers have been used for decades to synchronize electrical activity of the heart. **(b) Vagus Nerve Stimulation (VNS)**—Electrodes are implanted in direct contact with the vagus nerve and have proven to be effective in the management of epilepsy. **(c) Transcutaneous Electrical Stimulation(TENS)**– Low voltage electrical stimulation on the surface of the skin is used for the treatment of pain. **(d) Gastric Electrical Stimulation (GES)**—GES is used in cases of gastroparesis by delivering a high frequency electrical stimulation to the muscles in the stomach. **(e) Neuromuscular Electrical Stimulation (NMES)**—NMES is used in rehabilitation engineering to condition skeletal muscles for cases of muscle atrophy related to aging or trauma. **(f) Lower Limb Electrical Stimulation (LLES)**—LLES is a specialized case of NMES used for patients with chronic heart failure as a mechanism to increase muscle activity.

leads are inserted through a vein that runs under the collarbone. Since the electrodes are in direct contact with heart muscle, they monitor the electrical activity of the heart. If the electrical activity of the heart is normal, the control unit does not deliver any signal. However, if the electrical activity of the heart deviates from normal behavior, the electrodes are able to sense this change and deliver an electrical impulse that resynchronizes the cardiac conduction system. This process continues for every heartbeat. Therefore, cardiac pacemakers serve as sensors for cardiac electrical conduction and as on-demand delivery vehicles for electrical stimulation protocols.

Vagus nerve stimulation (VNS) is another example in which electrical stimulation has been used as a treatment modality, in this case for epilepsy (80–83) (Figure 6.9b). Epilepsy is a condition in which patients suffer from spontaneous seizures that vary in frequency, duration, and intensity. The effect of these seizures varies among patients, but in most cases, there is some loss of awareness, physical control, and/or alteration in communication. Seizures are caused by random electrical activity from neurons in the brain that lose their ability to act in a synchronized manner. The exact cause of epilepsy is unknown, though genetic and environmental factors are known to have a role. Pharmacological agents are commonly used to control seizures, and in severe cases of epilepsy, VNS can also be used as a treatment modality. The vagus nerve runs from the brain stem to the chest and abdomen area and is the target for VNS. In VNS, a control unit, similar to that for cardiac pacemakers, is implanted in the collarbone area. Electrodes are placed in direct contact with the vagus nerve and provide long-term electrical stimulation of the vagus nerve; this process has proven to be effective in controlling seizures in cases of epilepsy.

Transcutaneous electrical nerve stimulation (TENS) is a process by which low-voltage electrical stimulation is delivered to the surface of the skin as a therapy for pain relief (84–89) (6–9C). As in the previous two examples, a control unit is used to regulate the electrical stimulation protocol, and the output is via two leads attached to the site of delivery, which is a specific point on the surface of the skin. TENS is used extensively as a therapy for relief of muscle, joint, or bone pain, especially for lower back and neck pain.

Gastroparesis is a medical condition in which partial paralysis of the stomach results in delayed emptying of the stomach (Figure 6.9d). Muscle contraction in the stomach causes food to move through the digestive tract and is under the regulation of the vagus nerve. In gastroparesis, muscle contraction in the stomach is compromised, causing food to remain in the stomach for extended periods of time. This can affect a patient's nutrient absorption and can also lead to nausea and vomiting. In such cases, high-frequency gastric electrical stimulation (GES) is used to support muscle contractions in the stomach (90–93). As in the previous cases, a control unit is implanted in a subcutaneous pocket with electrical leads touching the surface of the stomach. High-frequency electrical stimulation of the muscle has been shown to reduce symptoms associated with gastroparesis.

Neuromuscular electrical stimulation (NMES) is a technique used extensively in rehabilitation engineering, which involves the use of electrical stimulation to

condition superficial skeletal muscle (94,95) (Figure 6.9e). As we have seen in all the cases before, NMES involves the use of a control unit and electrodes—in this case, the electrodes are positioned in contact with the skin superficial to the muscle of interest. NMES is used extensively in physical therapy and rehabilitation clinics in cases associated with muscle injury, trauma, or aging; the objective of this therapy is to preserve, restore, and/or improve skeletal muscle function. Lower limb electrical stimulation (LLES) is used for patients with chronic heart failure as a rehabilitation tool to increase skeletal muscle activity, which can complement pharmacological treatment modalities for these patients (Figure 6.9f).

Electrical Stimulation and Tissue Engineering—Our discussion so far has focused on looking at examples of electrical stimulation as a treatment modality for different conditions. We have looked at cardiac pacemakers, VNS, TENS, GES, and NMES. It should become abundantly clear that electrical stimulation has been extensively used as a treatment or therapeutic modality. There is an entire field known as functional electrical stimulation (FES), which is based on electrical stimulation for functional recovery and encompasses all of the examples that have been discussed. Our next objective is to move from this broad overview of electrical stimulation to a specific application involving 3D artificial tissue. We will study the role of electrical stimulation in guiding 3D artificial tissue formation and function, and we will examine the design and development of bioreactors to achieve this objective.

There have been several applications that study the effect of electrical stimulation in tissue engineering. Some examples include neural tissue engineering applications, the culture and differentiation of stem cells, and the development and maturation of 3D artificial heart muscle. We will look at these applications in the following sections.

In one study, PC-12 cells, obtained from rat pheochromocytoma in the adrenal gland, were plated on surface of a thin film of oxidized polypyrrole (PPy), which is a conducting polymer (96) (Figure 6.10a).

In this study, cells were expanded in culture and plated on the surface of the PPy film with a 24-hour attachment period. After this initial attachment period, cells were exposed to one of two electrical protocols, using a constant voltage of 100 mV for 2 hours or a constant current of 10 μAmp, also for 2 hours. After the 2-hour stimulation protocol, the cells were retained in culture for an additional 24 hours and processed for histological assessment. Electrical stimulation resulted in an increase in differentiation phenotype of PC-12 cells toward a neuronal lineage, as was evident by an increase in neurite length and an increase in cell spreading.

The previous study showed the positive effects of electrical stimulation on PC-12 on a 2D monolayer surface, while the current study was conducted in a 3D polyaniline (PANI) scaffold (97) (Figure 6.10b). Electrospinning was used to fabricate 3D PANI scaffolds with a fiber diameter of 112–189 nm and pore size in the range of 0.27–1.5 μm. The 3D scaffolds were placed in a 24-well tissue culture plate and seeded with nerve stem cells (NSCs) at a density of 1.5×10^4 cells/plate. After a 24-hour culture period, the PANI scaffolds with the NSCs were subjected to an electrical stimulation protocol consisting of 1.5V for 15, 30, and 60 minutes and then

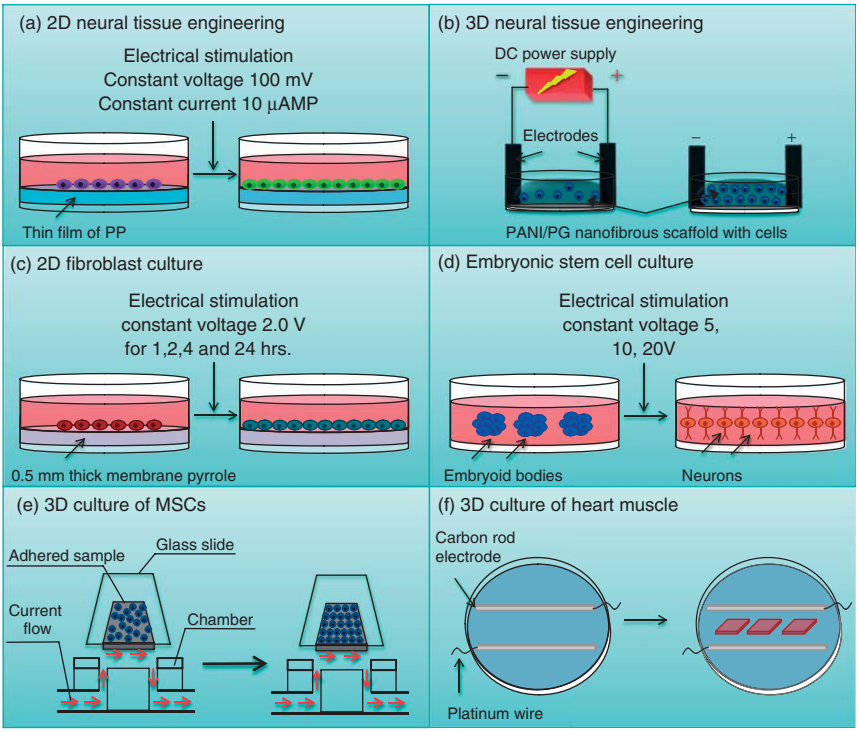

Figure 6.10 Bioreactors for Electrical Stimulation in Tissue Engineering—**(a) 2D Neural Tissue Engineering**—Differentiation of PC-12 cells toward a neural phenotype in response to electrical stimulation during 2D culture. **(b) 3D Neural Tissue Engineering**—Differentiation of NSCs toward a neural phenotype during 3D culture and in response to electrical stimulation. **(c) 2D Fibroblast Culture**—Increase in rate of fibroblast proliferation during 2D culture and electrical stimulation. **(d) Embryonic Stem Cell Culture**—Differentiation of ESCs toward a neuronal phenotype in response to electrical stimulation. **(e) 3D Culture of MSCs**—Increase in MSCs adhesion to substrate in response to electrical stimulation during 3D culture. **(f) 3D Culture of Heart Muscle**—Improvement in electrophysiological properties of artificial heart muscle in response to electrical stimulation.

maintained in culture for an additional 24 hours prior to histological assessment. As in the previous example, electrical stimulation resulted in an increase in neurite outgrowth, which was also accompanied by an increase in the rate of NSC proliferation.

In another study, a 0.5 mm thick membrane pyrrole was fabricated, and human skin fibroblasts were cultured on the surface of the membrane at a concentration of 6×10^4 cells/cm^2 (98) (Figure 6.10c). Immediately after cell seeding, an electrical stimulation protocol, which consisted of a constant voltage of 2V for 1, 2, 4, and 24 hours, was initiated. Electrical stimulation resulted in a significant increase in the rate of fibroblast proliferation, as measured by the MTT assay.

One interesting application of electrical stimulation has been to drive the differentiation fate of embryonic stem cells (99) (Figure 6.10d). ES cells were maintained in culture for 3 days to support formation of embryoid bodies (EB). Controlled electrical stimulation was delivered to the EBs after this initial 3 day culture period, and histological and functional assessment of the cells was conducted after another 10 days in culture. For electrical stimulation, a constant voltage protocol was used at 5, 10, and 20 volts while the cells were cultured in a 4 mm gap cuvette. This study demonstrated that electrical stimulation of ES cells resulted in differentiation of the cells toward a neuronal lineage. This is a very important finding since the ability to regulate the differentiation fate of ES cells toward any given lineage is a critical barrier that needs to be overcome prior to the utilization of these cells for therapeutic purposes.

Another interesting application has been the use of electrical stimulation for 3D culture of bone marrow-derived mesenchymal stem cells (100) (Figure 6.10e). MSCs were cultured with fibroblasts in a 3D collagen gel using a cell density of 2×10^5 cells/ml. The cells were cultured in the collagen gel for 12 hours prior to initiation of the electrical stimulation protocol. In order to deliver controlled electrical stimulation, a custom-fabricated chamber was used to house the 3D collagen gel with MSCs and fibroblasts, and a constant voltage of 7 V/cm was used. Electrical stimulation resulted in an increase in cellular orientation and an increase in the adhesion strength of MSCs to the collagen scaffold.

Electrical stimulation has been used extensively in cardiac tissue engineering applications to support the development and maturation of artificial heart muscle (101) (Figure 6.10f). Artificial heart muscle was fabricated by culturing primary neonatal cardiac myocytes within a 3D collagen sponge using 6×10^6 cells per sponge. The collagen sponges were maintained in culture for 3 days prior to electrical stimulation and then moved to custom bioreactors to initiate the stimulation protocol for an additional 5 days. The stimulation protocol consisted of square monophasic pulses with a duration of 2 ms, frequency of 1 Hz, and a variable voltage in the range of 0–12.5V. Electrical stimulation of artificial heart muscle resulted in a significant increase in cellular organization and alignment, as shown by histological data.

SUMMARY

Current State of the Art—Bioreactor technology is very well-developed and has broad applications in tissue engineering. Bioreactor technology has been incorporated in all facets of the tissue engineering process, from cell culture and expansion to physiological conditioning of artificial tissue. The current generation of bioreactors is advanced and has significant functional capabilities engineered within the system. During the course of this chapter, we have seen examples of bioreactor technology used to support fabrication of 3D artificial tissue and to deliver controlled stimuli to guide tissue formation and maturation. In addition, there is extensive literature that demonstrates a positive correlation between bioreactor conditioning and improvement in functional performance of 3D artificial tissue.

Thoughts for Future Research — There are three areas in the development of bioreactor technology: 1) bioreactors to simultaneously deliver multiple signals (electrical stimulation, stretch and perfusion), 2) bioreactors with embedded sensors for real time monitoring of artificial tissue function and using these signals for feedback control of bioreactor signals, and 3) bioreactors capable of regulating culture variables like temperature and pH without the need for cell culture incubators.

PRACTICE QUESTIONS

1. Explain the role of biomechanics during normal physiological function of mammalian tissue. Provide three specific examples, not described in the chapter, explaining the relationship between biomechanical forces and normal tissue function in mammalian tissue.

2. Discuss the role of bioreactors during the tissue fabrication process. What exactly are bioreactors and why are they important in tissue engineering?

3. Explain the difference between bioreactors for enabling technology and bioreactors for supporting technology. Identify any tissue or organ system that you would like to fabricate. Describe how bioreactors will be used to enable and support the fabrication of the selected tissue/organ.

4. How would the tissue fabrication process change in the absence of bioreactors? What impact would this have on the functional performance of 3D artificial tissue?

5. During our discussion of bioreactor classification, we provided six categories of bioreactors: bioreactors for cell culture, bioreactors for scaffold fabrication, bioreactors for scaffold cellularization, bioreactors for stretch, bioreactors for perfusion, and bioreactors for electrical stimulation. Explain each of these categories.

6. During our discussion of bioreactor classification, we provided six categories of bioreactors: bioreactors for cell culture, bioreactors for scaffold fabrication, bioreactors for scaffold cellularization, bioreactors for stretch, bioreactors for perfusion, and bioreactors for electrical stimulation. Identify any tissue or organ system that you would like to fabricate. For the selected tissue/organ, explain how these six categories of bioreactors will be used to support the tissue fabrication process.

7. We provided a process flow chart describing the design of bioreactors. The following variables were discussed: definition of the specific stimuli, identification of culture variables, development of sensor technology, and development of a specific stimulation protocol. Explain each of these steps.

8. We provided a process flow chart describing the design of bioreactors. The following variables were discussed: definition of the specific stimuli,

identification of culture variables, development of sensor technology, and development of a specific stimulation protocol. For this question, we will focus on the fabrication of artificial heart muscle; apply the process flow chart to 3D artificial heart muscle development. Explain how each of these four steps will be applied during different stages of the tissue development; end your discussion by providing specific variables for each of the four steps in the bioreactor process flow chart as they apply to the development of artificial heart muscle.

9. During our discussion of idealized bioreactors for artificial heart muscle, we described many critical components of the system. Explain the idealized bioreactor system for the culture of 3D artificial heart muscle.

10. During our discussion of idealized bioreactors for artificial heart muscle, we described many critical components of the system. Pick any tissue engineering application and develop an idealized bioreactor system for your selected application.

11. During our discussion of the integration of bioreactor technology with tissue engineering, which is illustrated in Figure 6.5, we described points along the tissue fabrication pathway where bioreactor technology has been incorporated: cell culture, scaffold fabrication, scaffold cellularization, and physiological conditioning. While this list encompasses many aspects of the tissue fabrication process, it is not exhaustive. There are several additional areas during the tissue fabrication process where bioreactor technology will be beneficial. Identify and discuss one such area where you believe that bioreactor technology will benefit the tissue fabrication process.

12. We discussed the potential use of bioreactors for the culture and expansion of mammalian cells. Compare the relative advantages and disadvantages of manual versus automated cell culture techniques.

13. Explain the process of electrospinning for scaffold fabrication. What variables affect the scaffold fabrication process?

14. During our discussion of bioreactors for scaffold cellularization, we looked at the use of direct cell transplantation and five additional strategies: cell entrapment using hydrogels, perfusion seeding, surface acoustic waves, centrifugal force, and magnetic nanoparticles. Each one of these technologies has relative advantages and disadvantages. Direct cell transplantation is extensively used to support the scaffold cellularization during the tissue fabrication process. Do you believe that direct cell transplantation is the most effect strategy for scaffold cellularization? Explain your answer.

15. During our discussion of bioreactors for scaffold cellularization, we studied six methods used for the cellularization of 3D scaffolds. Describe these six methods and discuss the relative advantages and disadvantages of each.

16. During our discussion of bioreactors for scaffold cellularization, we studied six methods used for the cellularization of 3D scaffolds. Select any tissue engineering application and explain which scaffold cellularization method is best suited for your application.

17. We discussed the role of perfusion systems to support the culture of 3D artificial tissue systems. Perfusion systems can be designed to operate within or independent of a cell culture incubator. What are the relative advantages and disadvantages of the two approaches?

18. What are some important processing variables that need to be controlled during perfusion of 3D artificial tissue? How would you control these processing variables?

19. We discussed the role of perfusion systems to support the culture of artificial tissue systems. Pick any tissue/organ system and develop a perfusion system to support the culture of the artificial tissue/organ. Explain why perfusion is important for your selected application. Describe what components will be parts of your perfusion system. Provide a schematic of your perfusion system. What will be your test variables? How will you identify optimal fluid flow conditions? End your discussion by providing a list of specific perfusion variables that you will use to support the culture of your selected tissue system.

20. Explain why stretch is important for the development and maturation of 3D artificial tissue.

21. How do cells within the cardiovascular system sense and response to stretch in the external culture environment?

22. What design variables are important for stretch bioreactors?

23. We discussed the role of stretch to support the culture of artificial tissue systems. Pick any tissue/organ system and develop a system to deliver controlled stretch to support the culture of the artificial tissue/organ. Explain why stretch is important for your selected application. Describe what components will be a part of your stretch system. Provide a schematic of your system. What will be your test variables? How will you identify optimal stretch conditions? End your discussion by providing a list of specific stretch variables that you will use to support the culture of your selected tissue system.

24. We have discussed the role of electrical stimulation to support the culture of artificial tissue systems. Pick any tissue/organ system and develop a system to deliver controlled electrical stimulation to support the culture of the artificial tissue/organ. Explain why electrical stimulation is important for your selected application. Describe what components will be a part of your system. Provide a schematic of your system. What will be your test variables? How will you identify the optimal electrical stimulation conditions? End your discussion

by providing a list of the specific electrical stimulation variables that you will use to support the culture of your selected tissue system.

25. During the course of this chapter, we have extensively discussed the role of bioreactors during the tissue fabrication process. All of our applications have been focused on the use of bioreactors *in vitro*. However, bioreactors are also used extensively *in vivo* during the tissue engineering process. Discuss how bioreactors can be used *in vivo*. How will bioreactors be used *in vivo* and what role will these *in vivo* bioreactors serve during the tissue fabrication process?

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