CHAPTER 13

Bioreactors: enabling technologies for research and manufacturing

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13.1 Learning objectives

After reading this chapter you will be able to:

- Describe general components of a bioreactor and a general bioreactor set-up.
- Identify crucial culture parameters that influence cellular behavior during bioreactor culture.
- Explain why it is necessary to mimic physiologic conditions and how they can be implemented by bioreactor technology.
- List different groups of bioreactor systems for cell expansion, production of cell-based products, and tissue engineering applications.
- Choose suitable bioreactors and monitoring systems for a specific expansion or tissue engineering process.

> By enabling reproducible and controlled changes of specific environmental factors, bioreactor systems provide both the technological means to reveal fundamental mechanisms of cell function in a 3D environment, and the potential to improve the quality of engineered tissues.
>
> **I. Martin, 2004**

> Bioreactors are the key to translate … tissue-based constructs into large-scale biological products that are clinically effective, safe and financially pliable.
>
> **J. Zhao, 2016**

13.2 Introduction

Traditionally, the development and establishment of production processes for biopharmaceuticals is considered as bioprocess engineering and encompasses all considerations regarding the up- and downstream aspects of a **bioprocess**. In this context, bioreactors are a central element as they assign the culture parameters and allow for a tight process control and therefore highly reproducible processes and products. From this perspective, the engineering of a tissue is likewise understood as a bioprocess for the production of a specific 3D cell-scaffold construct. To produce tissue engineered cell-scaffold constructs for

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in vitro models or in vivo applications, it is central to gain sufficient control on this process. This includes monitoring and control over all relevant culture parameters and preferably a high degree of automation.

In pharmaceutical bioprocesses, bioreactors are technical devices for the controlled aseptic culture of cells and manufacturing of the product. In this context, a bioreactor represents a **closed system**, a culture vessel (i.e., tank, bottle, chamber, etc.) with a medium inlet for nutrient supply and optionally a medium outlet for waste removal. During bioreactor development, there is already a high tendency toward up-scalability to be considered for effective mass production. The bioreactor is equipped with sensors to monitor **culture parameters** (i.e., temperature, CO_2, O_2, pH, etc.) and the necessary hardware and control systems to keep these parameters stable within feedback loops (heating, gas mixer, etc.). However, bioreactors for tissue engineering are thought to represent a simplification of the in vivo conditions of the target tissue. Thus, it appears that bioreactors for tissue engineering are clearly different from traditional pharmaceutical bioreactors in their appearance and functionality.

The main difference is that tissue engineering devices are designed to host a scaffold with cells mostly focusing on the representation of in vivo conditions instead of scalability and high throughput. Further, tissue engineering bioreactors comprise mechanical actuators that allow for the transduction of mechanical forces (shear, compression, strain, pressure) to the cells which support the maturation of a functional tissue. Compared to pharmaceutical bioreactors, this often results in rather complex bioreactor set-ups. Early systems filled an entire cell culture incubator, hosting only a single cell-scaffold construct.

In this chapter, we will give an overview on the basic components necessary to design a tissue engineering bioreactor, basic bioreactor set-ups, and introduce relevant sensors which will be necessary to monitor the bioprocess. Further, we will consider mass transport and physical stimuli to mimic physiologic culture conditions in a bioreactor system. Finally, we will give an overview on bioreactors for cell expansion and production of cell-based products and bioreactors for the controlled differentiation of cells.

13.3 Basic requirements

A multitude of bioreactors with various functionalities have been developed which makes it impossible to cover all possible set-ups and components. However, in this section we describe the minimal configuration that many tissue engineering bioreactors share to highlight the basic requirements. In general, bioreactors consist of a vessel, including a mount for the cell-scaffold construct. This vessel is connected to a medium reservoir and waste container via tubing and pumps. In many applications, a waste container is omitted and the medium is circulated from and to the medium reservoir (Fig. 13.1). Further, the bioreactor can be equipped with sensors. The entire set-up is operated in an incubator to ensure constant temperature and atmosphere. Although the incubator itself is not considered as a bioreactor component, it contributes to achieving vital culture conditions.

Since there is no common approach for the bioreactor chamber and the scaffold mount to fit all types of tissues, the material, the tissue architecture, and the geometry have to be adapted to the specific needs. Individual aspects of cell and developmental biology as well as geometrical functionalities of most tissues demand an individual set-up of physical stimuli. However, all components that are in contact with cells and/or the medium, especially the scaffold mount and the bioreactor vessel, must be inert,

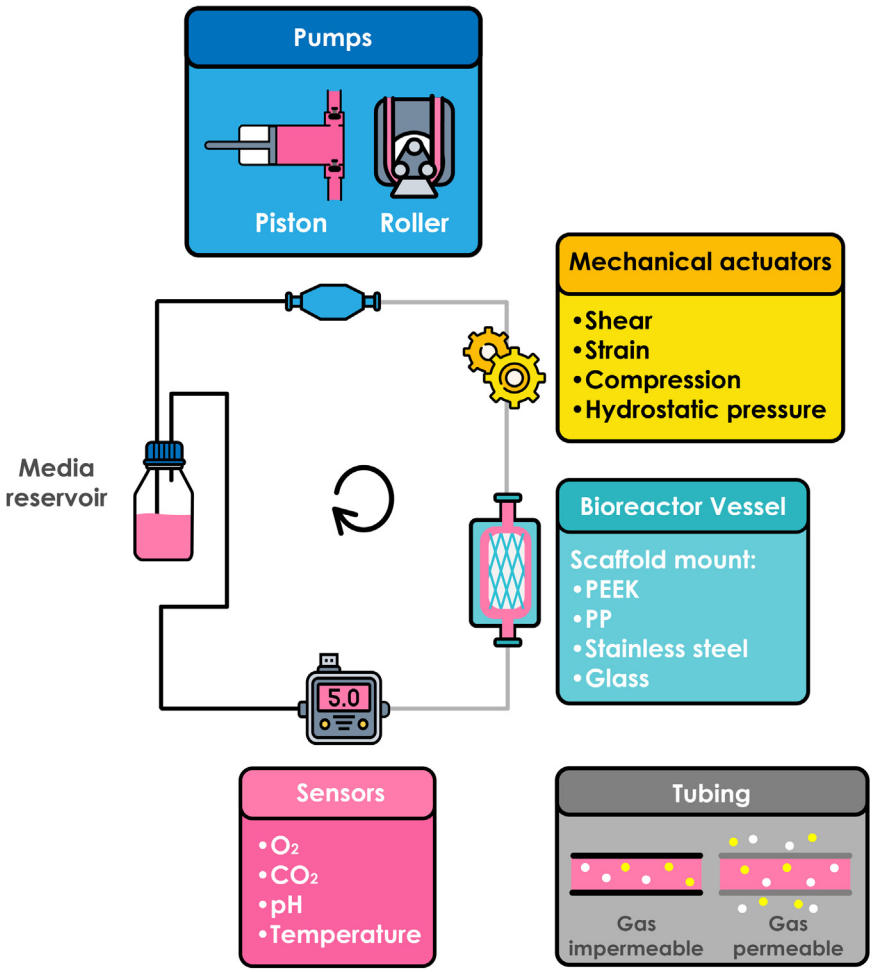

FIGURE 13.1
Circular bioreactor set-up with basic bioreactor components.

noncytotoxic, and preferentially **biocompatible**. If the bioreactors are supposed to be reusable, these materials must further be repeatedly sterilizable. Steam sterilizable materials which are often used are stainless steel, glass, polyether ether ketone or polypropylene. If other methods for sterilization are available (UV-exposure, gamma-irradiation, gas sterilization), virtually all biocompatible materials might be used. Further, 3D printing for rapid prototyping of bioreactors is becoming popular and some biocompatible resins are already available. The shape of bioreactor vessels and scaffolds mounts varies extremely depending on the targeted tissue and the scaffold material and size. Thus, countless variations exist which cannot be covered here. Instead, we refer to Chapter 11. The vessel itself is often equipped with mechanical actuators such as valves or linear motors to translate mechanical forces to the cell-scaffold construct.

Tubing is a crucial part of every bioreactor as it transports the media to and away from the cells. As the culture system should not be affected by unwanted external factors, the tubing must be inert and biocompatible. However, repeated steam sterilization or the use of harsh bases or acids can promote the transfer of leachables from the tubing material into the media with unknown effects on the process. Further, tubing is available from gas permeable materials such as widely used silicone tubing or gas impermeable, such as fluoroelastomers. Thus, the tubing material must be chosen carefully according to the respective bioprocess, cleaning procedures, and sterilization method.

As pumps transport the media through tubing, they are an integral part of a bioreactor system. Pumps for a bioreactor system are necessary to apply a constant or pulsatile flow and must operate at a high precision for several weeks. Peristaltic pumps, also called roller pumps, are the most widely used devices. The tubing is mounted on a rotor with several rollers and the liquid is displaced by rollers through a rotary motion. However, any free-floating cells are squeezed by the rollers which might lead to unwanted loss of cells or other side effects. Thus, piston pumps represent a viable option as any floating components experience less shear forces.

The bioreactor vessel with media reservoir and tubing is placed in an incubator with stable temperature and atmosphere. To ensure constant pH over the culture process, media contain buffer systems that require supply of 5% CO_2 to function properly. Further, in some differentiation processes, an oxygen-reduced atmosphere is necessary. For this, special incubators are available that can reduce the oxygen concentration by adding nitrogen to the atmosphere. Even more advanced incubators are available which are equipped with pumps, sensors, feedback control, documentation systems, and even mechanical actuators for transduction of mechanical forces to the cells (Fig. 13.2).

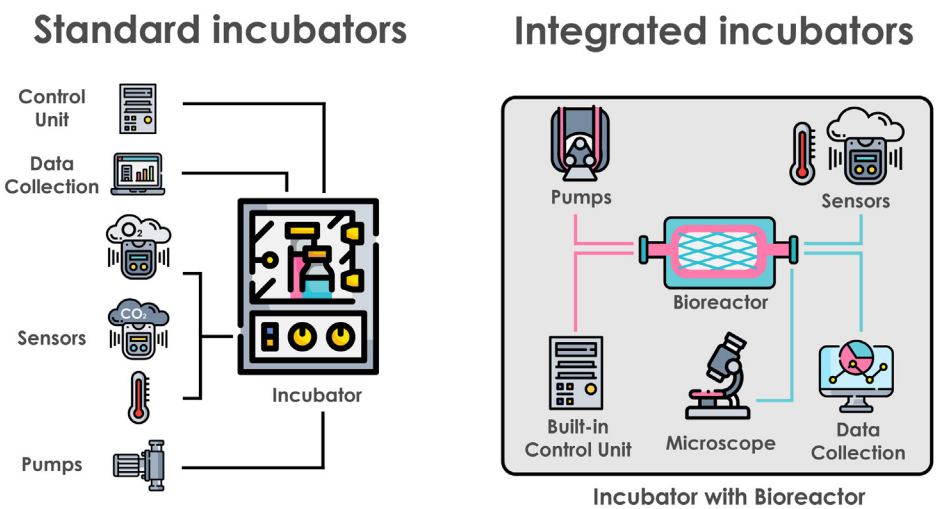

FIGURE 13.2

Traditional standard incubators versus advanced integrated incubator systems.

In addition, bioreactors should be equipped with sensors to enable monitoring and control of crucial culture parameters. These parameters can vary, depending on the culture process. However, temperature, atmospheric CO_2, dissolved O_2 and pH must be monitored and controlled in almost all bioprocesses. Biomass, pressure, impedance, glucose, and ubiquitous metabolites such as lactate are parameters that can be of further interest in tissue engineering. Optimally, sensors should measure on-line and **noninvasively**. Most of the available sensors measure in the circulating media and not in the 3D structure itself although the target parameter might be highly diluted in the media, compared to the inside of the cell-matrix construct. Therefore, noninvasive on-line monitoring of crucial parameters inside the 3D cell-scaffold is urgently needed but remains challenging. If suitable sensors are available, their data can feed a control system which can regulate and document the bioprocess. This can greatly contribute to the robustness and finally the success of a tissue engineering process.

13.4 Mimicking physiological culture conditions

13.4.1 Mass transport

When a tissue is cultured in vitro, a basic requirement for cell viability and development is sufficient **mass transport**. Mass transport fulfills three functions: First, nutrients are delivered to the cells. Second, waste products are removed. Third, information between cells is exchanged via soluble factors (see Chapter 14). Hereby, diffusion is the primary driving force but also the most limiting factor. For example, for a tissue stored under static conditions submerged with cell culture medium: Here the medium serves as a **source** of nutrients such as glucose and growth factors and constitutes a **sink** for waste products (see Chapter 4). Due to a difference in concentration between the supernatant and the tissue, a flux of various factors is established. Inside the tissue, the magnitude of the flux is governed by the diffusion coefficient D and the concentration gradient of the respective molecule (Eq. 13.1). The direction of this diffusive flux is orientated against the concentration gradient—from higher to lower concentrations. During culture, a time-dependent concentration profile is detected. Considering a nutrient concentration in the tissue, the highest value is found at the interface between supernatant and tissue (Fig. 13.3a). With increasing the distance from the supernatant-tissue interface toward the core of the tissue, the nutrient concentration decays (Fig. 13.3c). The reason for this is consumption by cells and diffusion limitations. To model the temporal and spatial development of the concentration pattern, a combination of Fick's second law of diffusion and a reaction term can be applied (Eq. 13.2). Most importantly, the concentration at the supernatant-tissue interface decreases over time. This results in a lack of nutrients inside the tissue. The duration from the initial inflow of nutrients across the supernatant-tissue interface until its depletion depends on the diffusion coefficient and the half-life/**elimination rate**. Assuming a diffusion coefficient of 1.42×10^{-11} m^2/s (in the medium) and 1.1×10^{-11} m^2/s (in the matrix), which are typical for proteins, and a consumption rate of 6×10^{-5} 1/s (half-live of approximately 3 h 15 min), the clearance of a factor can already happen 30 h after medium exchange.[1] Additionally, the forming of a gradient in the supernatant limits the flux of nutrients into the tissue. In vivo, the tissue is supplied by the vasculature that distributes

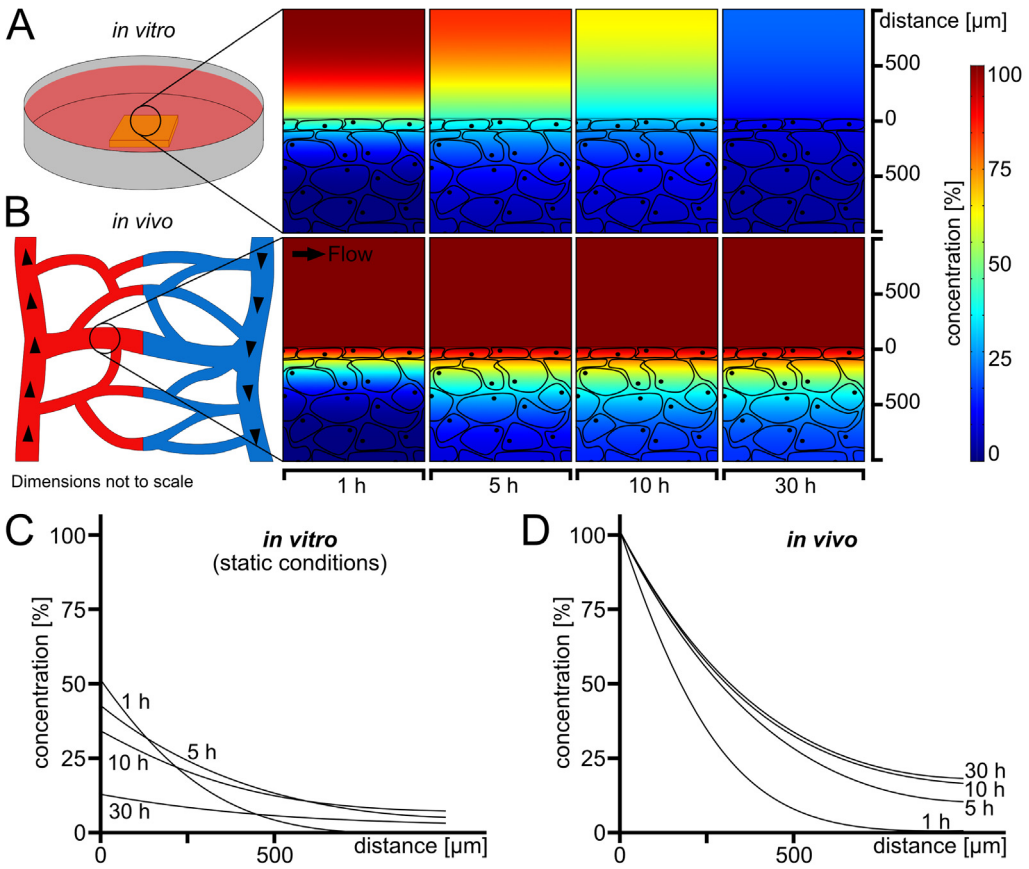

FIGURE 13.3

Concentration gradients of a growth factor within a simple in vitro set-up (static) and in vivo.

nutrients and removes waste products very efficiently. In contrast to the in vitro culture conditions, the blood stream maintains consistent nutrient concentrations at the blood–tissue interface. When assuming constant boundary conditions for a nutrient, a steady concentration profile is established (Fig. 13.3b and d). However, even in vivo, the concentration profile shows a limited range into the tissue. This again depends on the diffusion coefficient and the **consumption rate**. To overcome this limitation, capillaries from the vasculature spread throughout most tissues except cartilage, epidermis, and cornea. Considering waste product removal, the blood serves as sink with low waste product concentrations, thereby enabling full depletion of the tissue from metabolites. Communication via soluble factors is also based on diffusion and elimination; nevertheless, cells are the growth factor releasing source, as well as the growth factor binding sink of the tissue (see Chapters 11 and 14).

In the following, the calculation of mass diffusion and shear stress are listed:

Fick's first law

$$\vec{J} = -D\nabla\varphi \quad (13.1)$$

$\vec{J}$ = Diffusion flux (amount of substance per surface and per time), D = Diffusion coefficient in m^2/s, and φ = Concentration (amount of substance per volume).

Fick's second law with reaction term

$$\frac{\partial\varphi}{\partial t} = D\Delta\varphi - k\varphi \quad (13.2)$$

φ = Concentration (amount of substance per volume), D = Diffusion coefficient in m^2/s, and k = elimination rate (amount of substance per time).

The comparison between static in vitro culture and the in vivo situation reveals basic design principles for proper nutrient supply and waste product removal. In order to improve in vitro culture conditions, the volume of the cell culture medium should be chosen very carefully. Importantly, too low volumes result in rapid depletion of nutrients; too high medium volumes can entail negative effects for cells: For example, released soluble factors are diluted in the supernatant and thereby impairing cellular crosstalk. To ensure high flux into and from the tissue, the boundary conditions at the supernatant-tissue interface should be controlled. This can be achieved by dynamic in vitro culture using **perfusion bioreactor** systems which are described in detail later in this chapter.

According to these design principles, perfusion-bioreactor set-ups are harnessed to optimize nutrient supply. Moreover, strategies to embed tube-like structures into a tissue have been developed. The shape and geometries of a bioreactor system show a highly linked development with vascularization approaches. Tools for proper vascularization could be on one hand the generation of channels or vessels by casting, sacrificial structures, 3D bioprinting, postprocessing, or else. On the other hand, porous scaffolds can be used for full perfusion of the tissue graft. Additionally, prevascularized scaffolds have been used for a long time like decellularized intestine, pancreas, or else. The reseeding of those ex vivo structures is promising but do lack geometrical freedom (see Chapter 14).

13.4.2 Physical stimuli

Besides mass transport, many additional factors affect cell viability and development. (see Chapters 3 and 4). Even though most stimuli have more than one effect on the tissue, they can be roughly categorized into distinct types of stimuli (Fig. 13.4): First of all, cells communicate via cell–cell contacts or through secreted signaling molecules. Moreover, the parameters of the matrix or scaffold like stiffness affect the cell behavior. Cells have the ability to remodel the matrix and/or can produce their own extracellular matrix proteins (see Chapters 5, 7, and 8 for more details on ECM and cell–material interactions). The next category of stimuli is provided by the medium: Physical stimuli like shear stress as well as biochemical stimuli like pH, nutrition, CO_2, and many more are provided via the culture medium. Last, there are stimuli like temperature, strain, or electrical treatments that are applied system wide.

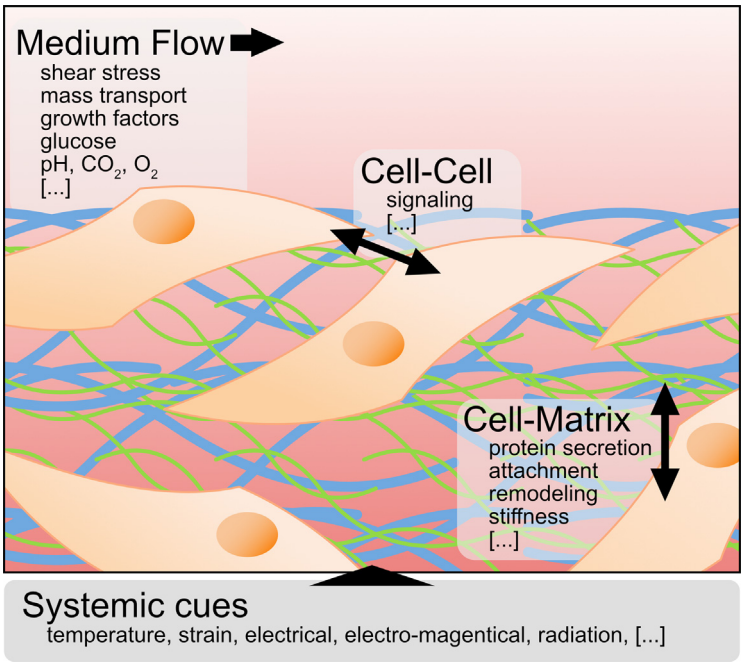

FIGURE 13.4

Selection of physical stimuli with impact to the tissue and tissue maturation within an in vitro bioreactor system.

To suit specific requirements of each type of tissue, these interactions can be tuned to establish *in vivo*-like culture conditions. To guide tissue maturation, most relevant in vivo stimuli of the desired tissue have to be identified (Table 13.1). For example, for lung tissue it would be beneficial to simulate breathing, causing a periodic in- and out-flow of air and stretching of the tissue, as well as a liquid–air boundary layer. For vascularization, endothelial cells form a denser blood–tissue barrier when exposed to shear forces and periodic systolic and diastolic pressure. Nevertheless, a bioreactor set-up always renders a simplification of the in vivo situation, since an exact mimic is nearly impossible and/or uneconomical.

Even though novel bioreactors aim to be easily adaptable and exhibit universal geometries, current bioreactors tend to be single tissue only.

Table 13.1 Special requirements for different types of tissues.

Tissue	Simplified functionality	Possible stimuli
Lung	Breathing	Air–liquid boundary, stretching, air composition
Muscle	Motion	Stretching contraction, electrical stimulation
Bone	Stability	Strain, weight bearing
Vessels	Mass transport	Flow, pressure, shear stress
Skin	Barrier	Air–liquid boundary

13.5 Bioreactors for cell expansion and cell-based products

Both cell-based therapies and tissue engineered grafts require extremely large numbers of cells. For example, the average cell number per patient and dose of mesenchymal stem cells in current clinical trials is around 100 million cells and usually more than one dose is administered for maximum effect.[2] It needs an immense amount of time, space, and single-use laboratory plasticware if the cells were expanded in traditional 2D cell culture. Also, harvest of the cells and cell-derived products (i.e., growth factors or extracellular vesicles) is demanding, laborious, or not feasible in 2D cell culture. Hence, improved culture strategies are necessary to allow cost and time efficient expansion of cells in clinically relevant numbers. Several culture systems are commercially available and have been developed to enable specifically the manufacturing of cells. Culture systems that are used in tissue engineering for initiating differentiation and thus maturation of the graft, often by application of mechanical forces, are discussed later in this chapter. However, both types of reactors are part of the tissue engineering process. For example, chondrocytes can be expanded within an expansion bioreactor system in vitro to gain the necessary cell numbers for treatment; however, they rapidly dedifferentiate during this expansion. Yet, after the expansion phase, they can be redifferentiated by applying mechanical stimulation onto them via a compression bioreactor. The large-scale production of cell aggregates, spheroids, or organoids in expansion bioreactors is often the first step for many "macro sized" tissue engineering attempts, particularly used as part of bio inks in bioprinting.

Nevertheless, whether it is a tissue engineered, mature graft, cells, or cell-based products, it has to be evaluated in a standardized quality control protocol. Therapies that are already in clinical application are hematopoietic stem cell transplantations as a treatment of hematologic cancers, like natural killer cells, tumor-infiltrating lymphocytes, or modified cells like engineered T cell receptor cells, and chimeric antigen expressing T cells. For these purposes, commercial, good manufacturing practice (GMP) compliant automated or semiautomated bioreactor systems exist: CliniMACS Prodigy, DynaMag, and the G-Rex system. Still in the clinical trial phase are attempts to use stem cells for the treatment of, for example, spinal cord injury, ischemic stroke of the brain, burn wounds, heart infarction, graft versus host disease, diabetes, muscular traumas, and Parkinson's disease (Table 13.2).

13.5.1 Cell-based products

Recent developments have shown that many beneficial effects of administered cells could also be reproduced by just applying their secreted proteins, growth factors, and extracellular vesicles, which contain among others aforementioned proteins and peptides, but also mRNA. This could omit problems that arise with the transplantation of cells, like immunogenic responses or cancer formation. While cells for therapy require harvesting from the bioreactors, this is not always necessary for production of cellular products. In this context, bioreactors that enable prolonged culture of cells and allow for continuous harvest of the secreted target product are of interest.

13.5.2 Bioreactor types

Different cell types as well as different intended applications of those cells require different culture parameters. Therefore, a multitude of diverse bioreactor systems can be found on the market, with both strengths and weaknesses. The way in which cells can be cultivated is strongly influenced

Table 13.2 Overview on cells for cellular therapies.

Cell type	Cell number	Application	Culture	Phase	References
UCART123 (gene-edited T cells)	Not provided	Targets Cd123 antigen expressed on leukemic cells in acute myeloid leukemia (AML)	Suspension	Clinical phase I	Clinicaltrials.gov, (NCT03190278)
Placental MSCs (PLX-PAD)	2x–4 × 1.5 × 10^8 cells	Graft versus host disease	Adherent in PLX bioreactor system (perfusion reactor)	Clinical phase I/II	Pluristem.com
Placental MSCs (PLX-PAD)	1 × 1.5 × 10^8 cells	Muscle regeneration following hip fracture	Adherent in PLX bioreactor system (perfusion reactor)	Clinical phase III	Norgren et al.[3], Clinicaltrials.gov (NCT03451916)
Tumor-infiltrating lymphocytes (TILs)	4.4 × 10^{10} cells	Adaptive cell transfer treatment for metastatic melanoma	Suspension in WAVE bioreactor	In vitro	Somerville et al.[4]
hiPSC-derived cardiomyocytes	7.5 × 10^9 cells	Bioartificial cardiac tissue formation	Aggregate suspension in DASBox system	In vitro	Jiang et al.[5]
Adipose MSCs	-	Mass production of hMSCs for therapeutic and industrial applications	Aggregate suspension in stirred tank	In vitro	Egger et al.[6]
Human skin–derived precursor cells	50 × 10^6 cells/ 20 cm^2 wound	Spilt thickness skin graft	Aggregate suspension in stirred tank	In vitro	Surrao et al.[7]
iPSCs	3.6 × 10^6 cells/mL	Mass production of hPSCs for therapeutic and industrial applications	Aggregate suspension in DASBox system	In vitro	Kropp et al.[8]
Human embryonic stem cells (hESCs)	3 × 10^6 cells (mouse model)	Functional pancreatic progenitors for diabetes type I	Adherent in cell factory	In vitro (mouse model)	Schulz et al.[9]
Human-induced pluripotent stem cells (hPSCs)		Mass production of hPSCs for therapeutic and industrial applications	Aggregate suspension in dynamic mixing (BioLevitator system)	In vitro	Elanzew et al.[10]

hiPSCs, human-induced pluripotent stem cells; MSCs, mesenchymal stem cells.

whether they are adherently growing (need to attach to a substrate) or if they can be kept in suspension (for example, blood cells). Microcarriers, spheres with a diameter of 150–300 µm, made from glass, dextran, and synthetic (acrylamide, polystyrene) or biological polymers (alginate, collagen),[11] are used to cultivate adherent cells in traditional suspension bioreactors. Next to this, anchorage-dependent cells can also use each other as a substrate, in the form of cell aggregates. Here, the cells are allowed to attach to each other to form multicellular aggregates and produce their own support matrix. Enabling cells to grow in these more natural 3D shapes has gained attention in the last years as a more physiological cultivation strategy.[12,13] Furthermore, special bioreactor systems have designated surfaces/substrates directly integrated into the system designed to home adherent cells. Some examples of bioreactors are presented in Figs. 13.5 and 13.6, including stirred tank bioreactor, spinner flask, vertical wheel, hollow fiber (HF), and meandering perfusion reactor, and will now be further described in more detail.

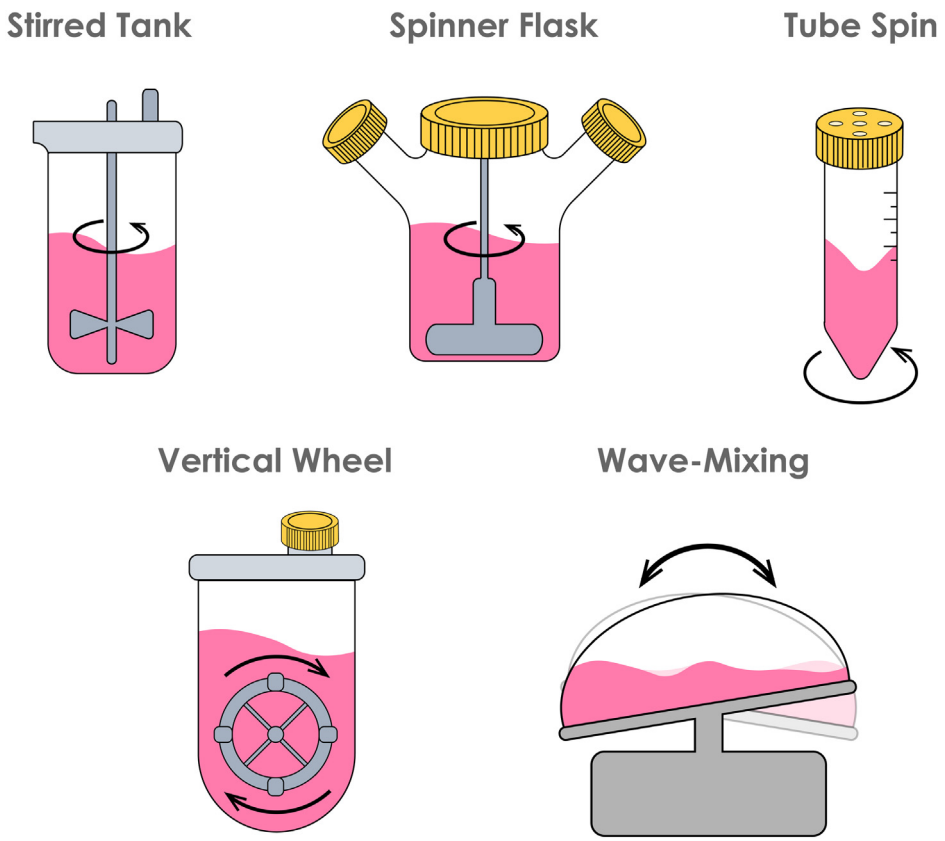

FIGURE 13.5
Mixing bioreactors.

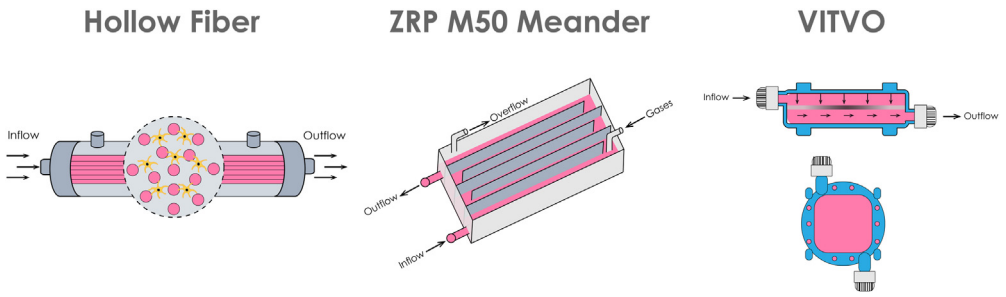

FIGURE 13.6
Perfusion bioreactors.

13.5.2.1 Mixing bioreactors

The most prominent group are **mixing bioreactors**. As their name implies, they homogeneously distribute nutrients, oxygen as well as cellular byproducts via stirring or oscillating and rocking components. Depending on cell type, they can be kept in suspension by mixing or if they need a substrate, grown on microcarriers, which in turn are kept in suspension. Mixing bioreactors can be categorized in three classes. The stirred tank bioreactors (ambr 250 modular—Sartorius AG, Mobius CellReady—Merck Millipore, BioBLU—Eppendorf AG) and spinner flasks (Corning Inc.) have a cylindrical or round bottom shaped vessel, respectively, with a vertical rotor or impeller. Vertical wheel bioreactors (PBS Biotech) are equipped with an upright paddle wheel that are driven by a physical connection, buoyant forces of gas bubbles sparged into the system, or by magnetic force. All three rotor-based mixing reactors are available commercially from multiple suppliers at research as well as industrial scales. The smaller benchtop bioreactors can often be placed in a standard incubator to control temperature and atmosphere, whereas larger bioreactors are equipped with their own, independent control systems. Cell growth is highly dependent on central properties like vessel geometry, aeration, and impeller geometry. Especially, the latter has great influence on mixing time, power input, and generation of shear forces. Therefore, attention must be directed at potential damage or modulation of the cellular differentiation potential. Here, the application of computational fluid dynamic simulation tools can give valuable information on peak forces and help narrow down the optimization range of the stirring speed where proper mixing and separation of the single microcarriers or cells is given but not too high to cause any adverse effects.[14]

Wave-mixing bioreactors use a flexible, gas permeable culture bag placed on a rocking platform to mix the liquid inside the bag, and represent the next large class of mixing bioreactors. Compared to most stirred systems, this is a rather gentle way of mixing with usually less shear forces and does provide a bubble-free aeration. Again, different sizes from 1 to 500 L are available.

Another example, that showcases how simple yet efficient a bioreactor system can be, are spin tubes. The components of these systems are disposable, cylindrical cell culture tubes with a conical bottom and ventilated caps that do not need any additional platform but are simply placed on an orbital shaker within a standard incubator. Cells or microcarriers are kept in suspension by the rotational motion of the shaker and can easily be harvested by centrifugation directly in the spin tubes. Different volumes from 10 up to 400 mL per tube are available.

13.5.2.2 Perfusion bioreactors

In comparison to the aforementioned mixing bioreactors, where distribution of oxygen and nutrients is achieved by homogeneous blending, in perfusion bioreactors, a directed flow is applied to the cells or cell-seeded scaffolds. Additionally to the supply with fresh medium, this flow can also translate distinct shear stress to the cells, a mechanical stimulus that is also used to induce differentiation or enhance release of growth factors or extracellular vesicles.

Examples of simple perfusion bioreactors are fixed- or packed-bed bioreactors. They comprise a culture vessel, tightly packed with microcarriers or other scaffolds for adherent cells, that is continuously perfused with fresh medium. With this strategy, a higher productivity can be achieved compared to culture in stirred tank bioreactors due to higher cell densities. However, scale up is more difficult for fixed-bed systems and consequently these systems have an upper limit of 10–30 L. Commercial examples include iCellis (PALL) or Pluristem's proprietary production system. The perfusion rate must be chosen carefully to ensure nutrient supply while retaining low shear forces. Fixed-bed bioreactors are already in use for cell therapies in clinical trials (Table 13.2, i.e., PLX-PAD Pluristem).

HF bioreactors represent another class of perfusion bioreactors. They consist of a bundle of parallel, semipermeable HFs assembled in a cylindrical cartridge. The characteristics of the fiber membrane-like permeability can be tailored to the intended application and is usually given as molecular weight cut off. Thereby, the transfer through the fiber membranes can be tailored to the application. This reactor type also allows for various flow regimes as well as cell seeding possibilities. Offering an intracapillary and extracapillary lumen, cells can be seeded on either or on both sides of the fiber membrane (Fig. 13.6) and allows for coculture of different cell types, where they can receive signals from each other and still retrieve them separately at the end of the culture. The two compartments can additionally be kept static or perfused. In addition, different speeds or patterns on the inside and outside of the capillaries can be established, making this system an extremely adaptable environment.

The ZRP culture system from Zellwerk is a closed system supporting the application of different culture vessels within the same platform, based on perfusion. For example, the horizontal bed reactor is a cylinder with cell carrier sheets on the inside. Cells can grow on both sides of each sheet, while being adequately supplied with medium by gentle circulation of the bed. In the same system, a meander reactor can be installed. This reactor is intended for the culture of nonadherent human cells and tries to mimic the flow in blood vessels. For this, meandering channels in a rectangular vessel are perfused. The vessel is cell repellent, and the main focus of the system is the culture of suspension cells (mainly HSCs), which reside on the vessel bottom while stimulated gently by laminar flow of the medium. The system is GMP compliant and can be used to produce cells for therapies; however, both vessel types are single use plastic and thus have to be replaced after each culture run.

Lastly, the VITVO (Rigenerand) bioreactor is a miniaturized 3D perfusion reactor that creates an in vivo-like environment in a closed system. It consists of a rectangular frame with two transparent oxygenation membranes, allowing gas exchange and visual access during the whole culture. Inside is a fiber-based matrix (thickness: 400 μm), composed of a synthetic polyester separating the culture chamber. Consequently, the two compartments are accessible for cell seeding and medium flow through an inlet and outlet on each side, respectively. Liquid is filling up one chamber first and enters the second chamber

after passing through the matrix. Therefore, cell seeding of the 3D matrix is uniform and easy by applying the cell suspension through the inlet, same for the medium exchange. Furthermore, if cells are prestained with a fluorescence dye, it can be directly viewed under a microscope. The simplicity of design ensures adaptability to common laboratory equipment which benefits academic and industrial research and development fields. This system is however rather designed for drug screening or initial testing runs, as it is not intended to be scaled up. However, due to its ease of monitoring, it can play an important role in developing patient-specific therapies or be used for continuous production and harvest of cell-based products like exosomes.

13.5.3 Scale-up versus scale-out

As already mentioned during the description of the different reactor types, there is great interest in transitioning from research scale to large/industrial scale production. This step is of great importance and highly critical during product development if the respective therapy should ever become a cost-effective, widely used treatment. Hence, it is central that large batches with high cell yields can be manufactured reproducibly. **Scaling up** also reduces the operator-dependent variability and therefore ensures product quality.

However, increasing batch sizes is not the solution for all cell-based therapies. Allogenic therapies use cells from another individual, preferably a young healthy donor, which are administered to a matching patient. In contrast, autologous therapies use the patient's own cells, which are optionally modified before expansion and readministration. This means allogenic cell products can be produced in large quantities and given as an off-the-shelf product to many different patients. However, autologous treatments are limited to one receiver, one lot, and one treatment dose. For these patient-specific treatments, a different strategy is necessary—"**scale-out**." Rather than larger volumes, parallel smaller manufacturing units are necessary to treat more patients (Fig. 13.7). Examples of such scale-out are the DASBox system by Eppendorf and the Cocoon platform by Lonza.

13.6 Bioreactors for tissue engineering

Now that the initial hurdle of acquiring a relevant cell number is accomplished, especially in tissue engineering, two aspects have to be considered for further graft/treatment production: (1) combining cells with the scaffold (hydrogels, porous materials, etc.) and subsequently (2) differentiation of the cells toward the target tissue.

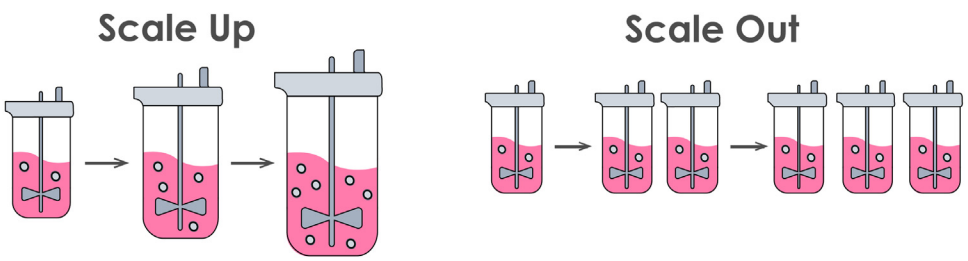

FIGURE 13.7
Scale-up versus scale-out approaches.

13.6.1 Cell seeding

Although it seems trivial, but before a graft can be differentiated first and foremost it has to be assembled. Often the initial cell seeding strategy has a major impact on the outcome of the tissue quality. Larger 3D constructs often have complex geometries, which can make uniform cell seeding challenging. With **dynamic seeding**, cells are introduced to the scaffold by an active motion of the seeding cell suspension. This can be achieved by centrifugal forces or rotation and spinning of the scaffold in the cell suspension (i.e., on a shaker platform). Another method is the utilization of pressure differential for seeding, which can be applied by a bioreactor system. Using a perfusion bioreactor can help to seed (rigid) porous scaffolds more evenly and effectively, also seeding within mixing bioreactors can enhance seeding efficiency. However, depending on the matrix to be seeded, higher cell densities at the surface than within the material were detected when random convection was used.[15] These techniques can greatly improve the penetration of cells into the scaffold and assure a homogeneous distribution, which is essential at the start of a tissue engineering process.

13.6.2 Differentiation

While expansion reactors are required to keep the cells in their initial, native state, bioreactors for tissue engineering are especially built to give the cells cues to induce maturation toward a specific lineage. Research from the last decade proved without any doubt that when biological, chemical, physical, and mechanical cues mimic the physiologic environment, cellular behavior changes dramatically. Mechanical forces in a physiological relevant range represent important environmental stimuli. Physical forces have a magnitude and a direction and can be distinguished between **shear**, **compression**, **strain**, and **hydrostatic pressure**. A combination of these results in bending or torsion (Fig. 13.8). Mechanical stress is an important factor for guiding cells during differentiation and can result in secretion of extracellular matrix molecules. Spatial organization of cells as seen in muscle and tendon or layered structures such as skin is a direct consequence of mechanical stimulation. In Table 13.3 a short overview of

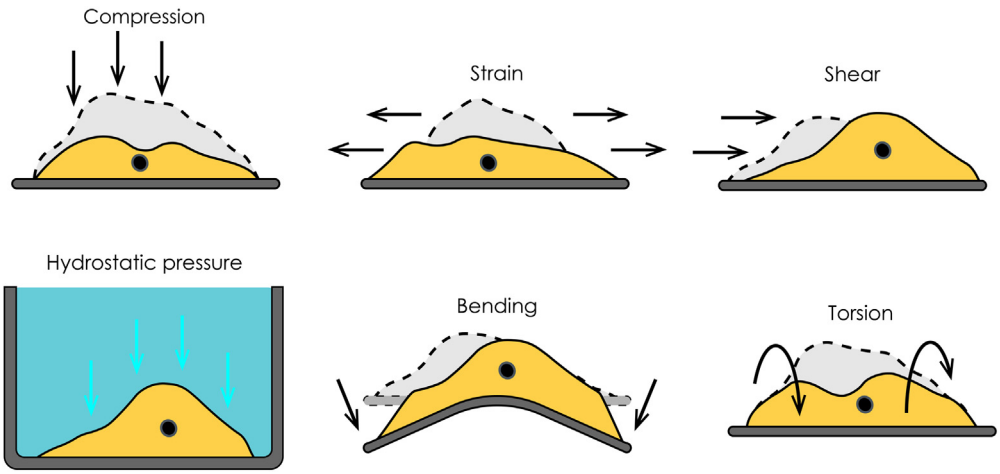

FIGURE 13.8
Mechanical forces acting on cells.

Table 13.3 Characteristics of mechanical forces in the human body.

Mechanical force	Induction of	Physiologic range	References
Compression	Chondrogenic differentiation	10–20 MPa	Hodge et al.[16]
Tension	Osteogenic chondrogenic, ligament differentiation	15–30 MPa	Schechtman et al.[17]
Hydrostatic pressure	Chondrogenic differentiation	5–6 MPa average, up to 18 MPa peak loading	Hodge et al.[16]
Fluid shear stress	Osteogenic differentiation	0.8–3 Pa	Weinbaum et al.[18]

the type of force, the resulting tissue formation, and in what range these forces are found in the physiological environment is presented.

Bioreactors for maturation of tissue engineering grafts are often custom built to fit the exact needs of a research project. However, there is a variety of bioreactors commercially available (Table 13.4 and Figs. 13.9, 13.10 and 13.11). The systems can provide the user with mechanical stimulation in the form of compression for cartilage engineering, tension especially for ligament and muscle engineering, hydrostatic pressure often used in chondrogenesis, and also fluid shear stress which can occur within vessels of the blood and lymphatic system and also used to improve bone formation. An overview of commercially available systems is presented in Table 13.4.

The TC3 by Ebers Medical is a versatile platform system with exchangeable scaffold mounts for different tissue engineering applications (Fig. 13.9a). All the chambers are perfused and the TC3 fits into standard incubator systems. Also, the company offers integrated incubator system with built-in peristaltic pumps. The U-CUP by Cellec Biotek AG is a U-shaped tube with a scaffold mount for porous scaffolds. Via a syringe pump, the medium in the tube can be driven back and forth which perfuses the scaffold

Table 13.4 Commercially available bioreactor systems for directed differentiation.

Mechanical stimulation	Commercially available bioreactor systems			
Compression	CartiGen[a]	FlexCell compression[b]		TC-3 bioreactor with compression or strain chamber[c]
Strain/Tension	FlexCell tension[b]	CartiGen[a]	LigaGen[a]	
Hydrostatic pressure	CartiGen HP[a]	TC3 bioreactor with hydrostatic pressure chamber[c]		
Fluid shear stress	InFlow perfusion bioreactor[d]	Streamer fluid shear stress device[b]	OsteoGen[a]	U-CUP[e]

[a] *By BISS TGT.*
[b] *By Flexcell.*
[c] *By Ebers Medical.*
[d] *By SKE Research Equipment.*
[e] *By Cellec Biotek.*

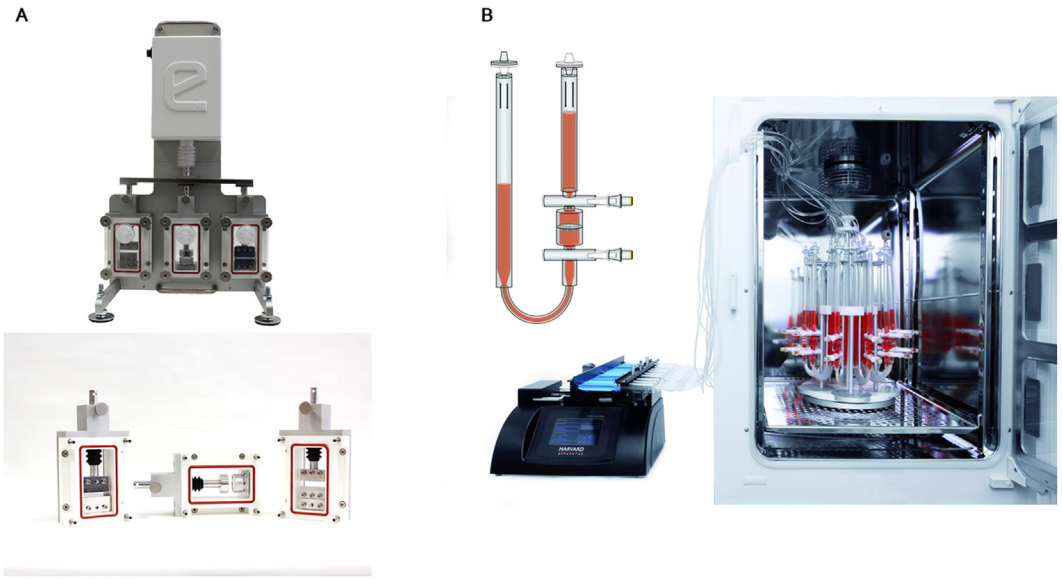

FIGURE 13.9
Commercial bioreactor systems. *From Cellec Biotek AG.*

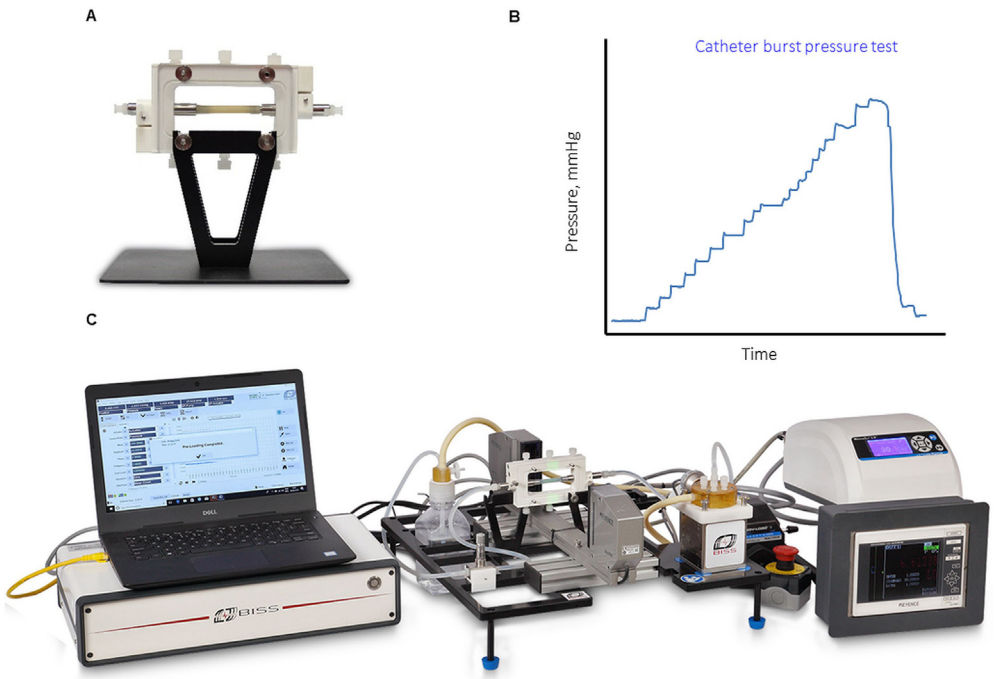

FIGURE 13.10
Commercial bioreactor systems. The LumeGen Pulsatile Stimulation Bioreactor System by ITW India.

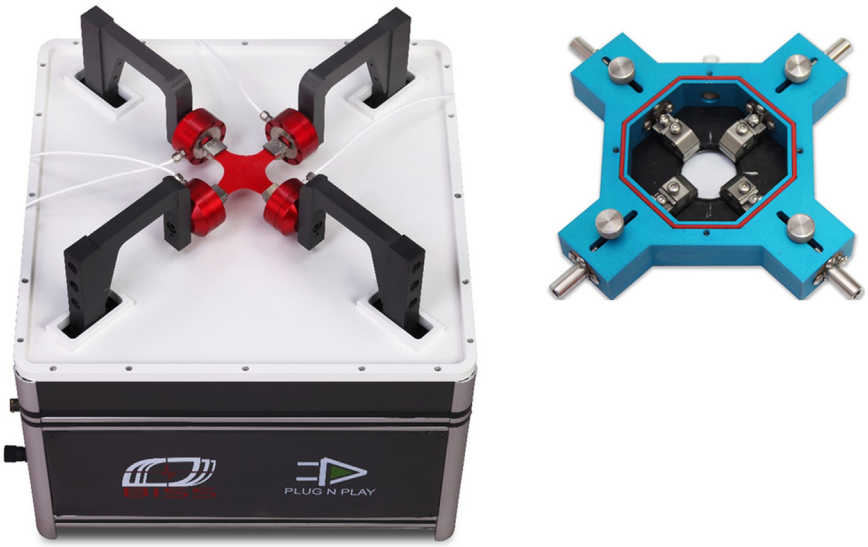

FIGURE 13.11
Commercial bioreactor systems. The planar bioaxial bioreactor system by ITW India.

and applies fluid shear stress to the cells. Two injection ports (up- and downstream the scaffold) can be used for dynamic cell seeing or sampling. The system can be parallelized so that up to 10 U-CUPs fit into one standard incubator system (Fig. 13.9b).

The company ITW India offers a range of bioreactor systems for the application of compression, strain, fluid shear stress, and hydrostatic pressure. The LumeGen Pulsatile Stimulation allows pulsatile stimulation of vascular tissue grafts, such as arterial grafts at physiological pressure waveforms. The vascular scaffold is mounted in a frame allowing for connection of the graft to a medium circuit (Fig. 13.10a).

Pulsatile stimulation in the LumeGen is achieved through a combination of the precise actuation mechanism and the GrowthWorks application software where the waveforms can be adjusted (Fig. 13.10b). LumeGen bioreactors allow luminal and transmural fluid flow (flow within blood or lymphatic vessels and flow from vessels to the surrounding tissue) to enable mechanobiological studies. These bioreactors have been used for development of arterial grafts and tissue engineered constructs. The LumeGen can be used with a CCD micrometer to measure real time strain as a function of pulsatile stimulation as well (Fig. 13.10c).

Another interesting system is the planar biaxial bioreactor system (also by ITW India). Here, a wide variety of specimens including PDMS sheets, thin films, and tissues can be gripped inside the bioreactor with standard fixtures. This allows for biaxial loading of the scaffold which is especially relevant in cardiovascular tissue engineering and for investigation of tissue viscoelastic properties (Fig. 13.11).

Often, the vessel or scaffold mount of commercially available systems is single use and needs to be replaced after use. On the one hand the use of established systems can save cost and time demanding development processes on the other hand these systems rarely allow for customization and can become expensive over time due to their single-use applicability.

13.7 Future perspective

The field of tissue engineering has experienced substantial advancements and has become a serious discipline with therapies that are already in application. The minimal configuration of a bioreactor comprises the vessel, a scaffold mount, tubing, pumps, sensors, and a suitable incubator system. The entire system should allow to overcome issues of mass transport, ensuring proper nutrient and gas supply through the entire 3D cell-scaffold construct. Further, a proper environment must be established with respect to the physiologic environment of the target tissue. For this, a proper matrix, but also physical stimuli, especially the application of mechanical forces, is required.

Bioreactor set-ups have evolved from large and complicated to smaller and smarter systems with integrated sensor technology that allow for in-process control. Although commercial bioreactor systems for the expansion and differentiation of cells are available, researchers still develop and improve bioreactor systems to enhance the maturation of cells toward a target tissue and to make tissue engineering processes more reproducible. For this, 3D printing in the context of rapid prototyping has become essential to allow for shorter development iterations. Although 3D printing of cell culture vessels and bioreactor parts is already possible, it will need more biocompatible resins in the future. Further, the sensor technologies that are available are mainly focused on measuring parameters in the cell culture supernatant. However, it will need more sensors that measure directly in the cell-scaffold construct, while being noninvasive, to ensure a high quality of the entire tissue. The translation of mechanical forces to the cells is required for a proper tissue maturation. Mathematical modeling can help to estimate these values, but a model is only as good as the underlying assumptions. Thus, sensors that measure the actual forces the cells are experiencing in the scaffold are necessary. This would greatly contribute to ensure the defined application of mechanical forces and thus monitoring and control of the entire process. In the context of industrial and medical applications, more bioreactor systems for the scale-out of tissue engineering processes must be developed to fulfill the future demand of tissue engineered constructs. For this, small simple bioreactor units and a high degree of process automation are required to ensure high-quality products. Again, the key is process control which can only be assured by more advanced sensor technology.

Summary

- Bioreactors for tissue engineering are thought to represent a simplification of the in vivo conditions of the target tissue.
- The minimal configuration of a tissue engineering bioreactor comprises a vessel, a scaffold mount, mechanical actuators, tubing, pumps, sensors, and an incubator.
- Monitoring and control of crucial culture parameters is essential for the development of a tissue engineering process.
- The size of a viable cell-scaffold construct is limited by mass transport of nutrients into and waste products out of the tissue. Thus, mass transport is the most crucial functionality of a bioreactor.
- The bioreactor requirements need to be evaluated for each target tissue separately. There is not "one-fits-it-all" solution to tissue engineering bioreactors.
- Besides the directed differentiation, the manufacturing of a relevant number of cells is an integral part of a tissue engineering process.
- Mixing bioreactors (stirred tank, spinner flasks, vertical wheel, wave) and perfusion bioreactors are the most important bioreactors for the manufacturing of large cell numbers.
- Dynamic cell seeding can greatly improve distribution of cells throughout the scaffold, which is essential at the start of a tissue engineering process.
- Mechanical forces (compression, tension hydrostatic pressure, fluid shear stress) are important stimuli to induce the differentiation of cells into the target tissue. Thus, bioreactors for the directed differentiation of cells are equipped with mechanical actuators to translate mechanical forces.

Classical experiment

Although the cornea is the most transplanted tissue worldwide, availability and quality of grafts are limited due to current methods of corneal storage. A dynamic bioreactor system enables the control of intraocular pressure and culture by air—liquid interface, mimicking the in vivo situation. In addition to a higher degree of tissue viability, healing of epithelial defects was achieved in this bioreactor. First, explanted cornea is fixed in the bioreactor system (Fig. 13.12a) consisting of a main body part that holds the tissue. A fixator keeps the cornea at its position. A lid with septum seals the bioreactor chamber. The septum is connected to one of the two separate medium circuits (Fig. 13.12b). Circuit 1 provides medium to the endothelial cells at the lower side of the cornea. Circuit 2 delivers medium to the epithelial side of the cornea to mimic tear fluid. The medium of this circuit is collected to a second chamber (bin) below the cornea tissue. Both circuits contain different mediums tailored to the individual cell types. Thereby, the cornea tissue is cultured for up to 7 days without a detrimental effect on the cornea.[19] In the future, additional sensors such as webcams, temperature sensors, pressure sensors, biochemical sensors like pH indicators, and many more can even increase the robustness of the culture process. To manage the acquired data, these sensor data can be analyzed by modern methods such as artificial neural networks to support smart control and error management.

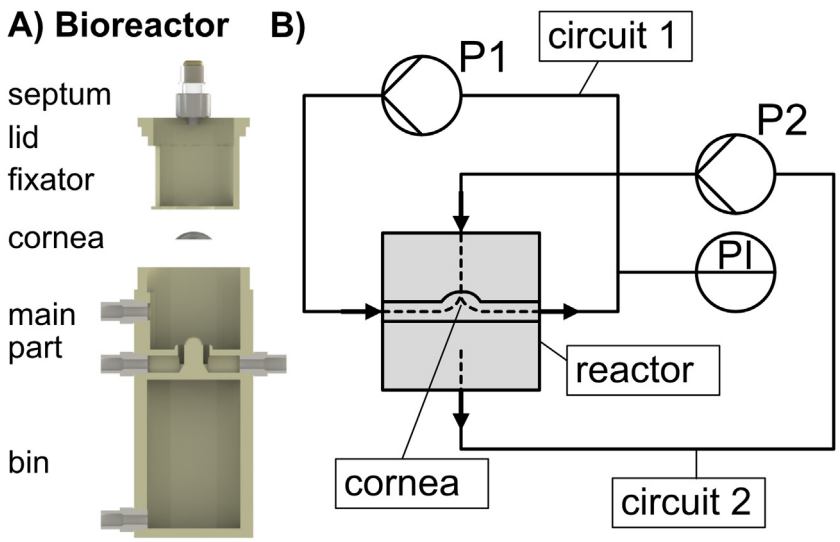

FIGURE 13.12

Bioreactor set-up for dynamic culture of native cornea tissue. *From Hansmann et al., 2017. Schmid, R., et al. In Vivo-like culture conditions in a bioreactor facilitate improved tissue quality in corneal storage.* Biotechnol J *2018;13(1):1700344.*

State-of-the-art experiment

In this state-of-the-art study, a miniaturized bioreactor for bone tissue engineering was developed and operated in a tailor-made integrated incubator system (Fig. 13.13a).[20] In the single reactor set-up, the bioreactor can be equipped with two pressure sensors (P) and a module for the application of hydrostatic pressure (HP; Fig. 13.13b). In the multireactor set-up, the parallel culture of up to eight bioreactors in one incubator is possible (Fig. 13.13c). With this system, the authors addressed the need for screening multiple culture conditions to optimize tissue engineering processes.

Human mesenchymal stem cells (MSCs) were seeded on a decellularized bone matrix and cultivated under static and dynamic conditions in osteogenic differentiation medium (ODM) and standard culture medium (CM). The dynamic conditions refer to the culture of a miniaturized perfusion bioreactor at a flow rate that induces fluid shear forces in a physiologic range to the cells. The shear forces were measured and controlled via noninvasive monitoring of the pressure differential over the scaffold. Interestingly, matrix mineralization, a marker of osteogenic differentiation, was observed in dynamic conditions only. Also, the mineralization in the control medium under dynamic conditions was superior than under static conditions with osteogenic induction medium (Fig. 13.13d). Further, thanks to the miniaturized and parallelizable set-up, several fluid shear stress regimes were screened and optimized. Also, the effect of hydrostatic pressure in combination with fluid shear forces on the osteogenic differentiation was assessed (Fig. 13.13e). For this, a hydrostatic pressure module was introduced to the incubator system and several pressure regimes were evaluated. Although the hydrostatic pressure did not further enhance the osteogenic differentiation of MSCs, the system proved its beneficial attributes for the screening and optimization of tissue engineering bioprocesses.

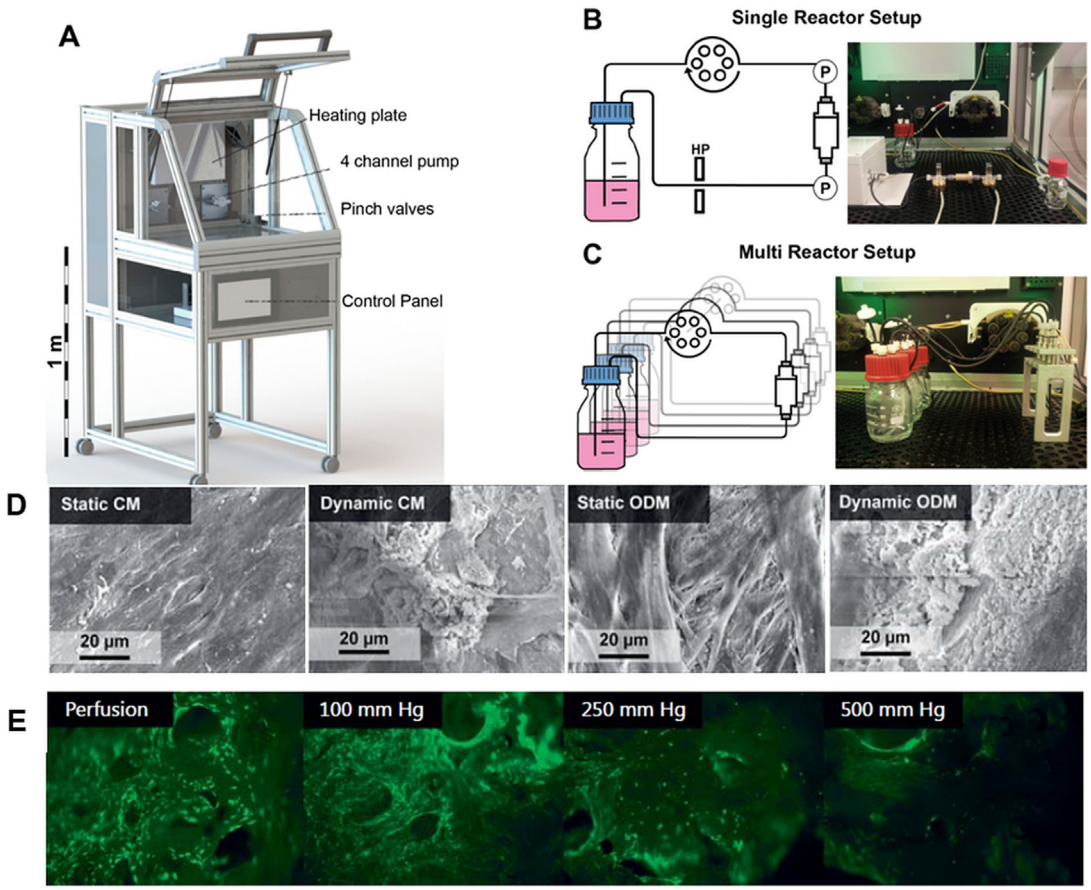

FIGURE 13.13

Integrated incubator with parallelizable miniaturized perfusion bioreactor system for screening and optimization of Tissue Engineering processes. *From Egger, D., et al. Application of a parallelizable perfusion bioreactor for physiologic 3D cell culture.* Cells Tissues Organs *2017;203(5):316—326.*

13.8 Recommended literature

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13.9 Assessment of your knowledge

(a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:

1. What compounds are necessary to set up a bioreactor system?
2. What do you need to consider when choosing the material for the tubing?
3. What are crucial properties of a sensor?
4. What are the two main classes of bioreactors for expansion of cells?
5. Why are scale-out strategies more relevant for tissue engineering applications rather than scale-up of a bioreactor?
6. How can you improve the cell distribution at the start of a tissue engineering process?
7. Which are the basic mechanical forces applied in tissue engineering?
8. Which forces are applied for the maturation of which kind of target tissue?
9. Is copper a suitable material for bioreactors?
10. How does perfusion increase the nutrient concentration within the tissue?
11. Which stimuli can be applied via the medium?
12. How is media perfusion applied in perfusion bioreactor systems and what other bioreactor systems are there establishing motion?

13. Is there a limit for tissue size and if so, what are the limiting factors and how could they be overcome?
14. Strain, weight bearing, and air–liquid boundary are the most critical stimuli for bone tissue engineering?
15. Argue whether commercially available bioreactor system are always superior to custom made systems.

(b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:

1. Describe a general tissue engineering process for the production of a bone substitute.
2. Why is it challenging to increase the size of a cell-scaffold construct?
3. Which stimuli could be important for generation of cornea tissue and how would a bioreactor look like?
4. How would you realize a microscopic real time observation of the tissue inside a bioreactor?
5. What are the main differences between bioreactors for bioprocess engineering and tissue engineering?
6. Describe physiological cell culture conditions.
7. Design a bioreactor system for a tissue engineered bladder and describe the requirements.
8. Explain Flick's laws and their application in tissue engineering.

Challenge-based learning

Continuous bioreactor to address graft versus host disease

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Human mesenchymal stem cells (hMSCs) have been a promising therapeutic cell source in tissue engineering over the past 15 years. However, hMSC engineering has proven to be more technically complicated than just extracting them, expanding them in vitro, and implanting them back into the patient. This is the reason why many therapeutic strategies with hMSC fail in clinical trials. Extensive research and preclinical/clinical validation are still necessary to make hMSCs a versatile, safe, and effective therapeutic tool. Human *allogenic* mesenchymal stem cells (haMSCs) have been engineered and cultured in bioreactors under highly controlled conditions to study their inherent immunosuppressive capacity. Results have shown that they can be promising therapeutic candidates in graft versus host disease (GvHD). This challenge's vision is to develop a stable culturing platform to generate readily available and effective immortalized MSCs for GvHD disease treatment.

Motivation and stakeholders

Human stem cells have the potential to become a tissue-engineered therapeutic tool in the future, but precise culture settings need to be established for each medical condition. To generate large numbers of cells, prototype bioreactor systems have been engineered in which cells are seeded onto polymeric particles, which are the free-floating scaffolds on which cells can proliferate. When new particles are added to the bioreactor, cells should move from a full particle to an empty one. This system can ensure continuous cell growth as long as the system is continuously fed with empty particles and the culture conditions are closely monitored. In standard 2D cell culture, confluency is manually monitored by direct viewing of a flask under a light microscope. At the moment, there is no automatic way to monitor the cell occupancy of particles in bioreactors to ensure nonstop growth of haMSCs. The design of bioreactors to grow hMSCs should consider the needs, requirements, and regulatory, financial, and

Continued

Challenge-based learning—continued

technical boundary conditions defined by stakeholders such as patients suffering from GvHD, medical doctors delivering cell-based therapies, stem cell biologists, and bioengineers who manufacture cell culture platforms.

Problem definition

In order to culture human allogenic MSC, nutrient level needs to be monitored in bioreactors. A proper model and design of a bioreactor to control haMSC's growth and metabolism would need to control the following variables: (a) proliferation rate, (b) nutrient availability and consumption rate, and (c) the rate at which new polymeric particles must be added to the bioreactor.

Challenge

To design a bioreactor system to produce a continuous source of immortalized human allogeneic mesenchymal stem cells as a therapeutic option to mitigate graft versus host disease. Pay close attention in the design of an automatic method to determine the particle occupancy percentage to ensure continuous cell growth.

Learning framework

Reading the Bioreactor chapter and related literature will help you to understand the following:

1. GvHD and the therapeutic potential of haMSCs to treat this medical condition.
2. The different types of bioreactors.
3. Methods to analyze bioreactor variables using sensors and probes.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

4. The parameters that need to be controlled in the bioreactor.
5. Existing biosensors to detect the cell's metabolic shift inside a bioreactor.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

13.10 Glossary

Biocompatible (or biocompatibility) is the ability of a material to perform its function with an appropriate host response without causing any undesirable local and systemic effects.

Bioprocess is a specific process that uses complete living cells or their components (e.g., bacteria, enzymes, chloroplasts) to obtain desired products.

Closed system is a physical system that does not allow transfer of matter in or out of the system.

Compression is the application of balanced inward forces to different points on a material or structure, to reduce its size in one or more directions.

Consumption rate is the average quantity of an item consumed or expended during a given time interval.

Culture parameters are a group of measurable factors that define a cell culture system or set the conditions of their operation.

Dynamic seeding uses agitation or perfusion of the cell suspension to actively increase cell seeding efficiency, uniformity, and/or penetration of cells into the scaffold.

Elimination rate is the rate at which a molecule is removed from a biological system.

Hydrostatic pressure is the pressure exerted by a fluid at equilibrium at a given point within the fluid.

Mass transport (in cell culture) is when materials are moved through the culture medium to the cell surface to deliver nutrients, remove waste, or trigger communication between cells through soluble factors.

Mixing bioreactors are a specific type of bioreactors that homogeneously distribute nutrients, oxygen, as well as cellular byproducts via stirring or oscillating and rocking components.

Noninvasive(ly) is not involving introduction of instruments in the culturing system.
Perfusion bioreactor is a continuous culturing method in which a directed flow of medium is applied to the cells or cell-seeded scaffolds.
Scale-out is adding more components for capacity expansion.
Scaling up is the development of culture systems in stages from (small scale) laboratory to (large scale) industry.
Shear (force) is the component of stress parallel to the material cross-section.
Sink is a reservoir that provides storage for a substance.
Strain is the opposite of compression. Thus, it represents a pulling force applied axially on an object which results in an increase of size in one direction.

Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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