Chapter 9
Bioreactors for Tissue Engineering

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Abstract Bioreactors have been widely used in various fields of biological production for many years. Their ability to provide a tightly controlled environment during the process and to allow for monitoring and intervention to the process parameters make them quite favorable to use in biological production lines. Also, bioreactors are widely employed in tissue engineering applications. Ideally, a tissue engineering bioreactor should have the capability to effectively regulate various environmental factors, such as pH, oxygen levels, temperature, nutrient transportation and waste elimination. Additionally, it should facilitate sterile operations, such as sampling and feeding, as well as automated procedures. The general approach for these applications include immobilization of suitable cells within porous, biodegradable and biocompatible scaffolds. These scaffolds serve as frameworks for tissue formation and the cell/scaffold constructs are cultured within a bioreactor, which creates a dynamic in vitro setting conducive to tissue growth. As the technology for these systems and required conditions continue to become more complex, these bioreactor designs will also evolve with time to help treat patients with diseases related to tissue damage. There are specific designs for various kinds of bioreactors (spinner flasks, rotating wall vessel bioreactors, perfusion systems, pulsatile systems, strain systems, hollow fiber systems, wave bioreactors, microfluidic bioreactors, compression and hydrostatic systems) in the market which allows better outcomes for certain applications

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such as cardiovascular tissue engineering, bladder tissue engineering, neural tissue engineering, cornea tissue engineering, kidney tissue engineering, musculoskeletal tissue engineering, lung tissue engineering and gastrointestinal tissue engineering. All of these different systems and their special applications for tissue engineering studies are explained in this chapter with their specific advantages and disadvantages which make them favorable with the physicochemical environment they provide. When current developments are examined and evaluated, it is seen that bioreactors will have enhanced designs that will help them better mimic the physiological pathways of cells, tissues and their interaction with the surroundings to have better solutions for whole organ, bone, and regenerative tissue engineering applications in the future.

Keywords Bioreactor · Scaffold · Tissue formation · Tissue engineering

Introduction

The term "bioreactor" is simply referred to a "device" or "system" designed to sustain biologically active conditions, which are necessary for cultivating an organism or conducting a biological reaction. Dating back to 1970's [149] bioreactors are being used for various fields of production including pharmaceuticals, fermentation products, and biotechnological products. Thanks to the ability of providing a tightly controlled environment and to be able to monitor the process closely as well as other design parameters for ensuring a reliable cell growth, they are still being widely used [58]. Various designs of disposable (single use e.g. spinner flasks) and reusable bioreactors (e.g. stainless-steel bioreactors) are present in the market with their distinct advantages and disadvantages for different biological productions process needs. When selecting the type of bioreactor that is going to be used for the production of a biological product, the selection between these bioreactors and their sub-choices (material, size etc.) are substantial to ensure a robust, efficient and reliable process [58]. When it comes to tissue engineering applications, just like any other process, bioreactors are designed to meet the needs of the cells to be cultured in them. Immobilization of cells within porous, biodegradable and biocompatible scaffolds allow them to be cultured within a bioreactor which enables tissue growth in a dynamic in vitro setting [23]. They can be divided into different categorized according to their detailed characteristics, such as the type of flow they have inside the chamber (laminar or turbulent) specific to the characteristic physiology of the tissues, or the type of pseudo-physiological environment they provide with a rotating or non-rotating designs [97].

In general terms, bioreactors are used in tissue engineering in order to mimic the natural physiological environment of cells to provide biochemical and physical regulatory signals, direct cells to differentiate and provide a suitable platform for the development of new tissues by stimulating extracellular matrix production [138]. These approaches enable the conditions for cells to be stimulated which enables the

production of the extracellular matrix (ECM) that is essential for vessel branching and vascular network formation [130].

Every system has their own unique advantages and disadvantages which qualify it feasible for specific applications and certain types of tissues. For instance, while stirrer bioreactors have an advantages in bone-tissue engineering applications by providing up-regulation of several growth factors, they reduce the diffusional gradients between the scaffolds, exposing cells to shear stress due to fluid convection [11, 144]. On the other hand, since shear stress is also beneficial for collagen and glycosaminoglycan (GAG) growth, such bioreactors may be useful for cartilage regeneration studies in cartilage-tissue engineering [12].

The simplicity or complexity of the system tends to change regarding their ultimate production goals. Factors such as the necessity for gas exchange, temperature control, how the access around the environment are going to be are some of the main examples that specify the complexity of system. Even though they differ for various applications, almost all of them have similar design parameters (maintaining the concentration of nutrients and gasses, providing mass transport, sustaining critical parameters such as pH and temperature etc.) that allow their usage for almost any biological production [136]. In addition to these basic factors, bioreactors have evolved from these designs with additional features to have different mechanical and biological components necessary to resemble the physiological conditions of the tissue's environment [121]. Advanced studies on this matter and providing new production solutions will make a great impact on improving the health of people with similar conditions.

In this chapter, some of the most common types of bioreactors ranging from basic designs to complex systems will be evaluated in terms of their advantages and disadvantages along with their critical design parameters, applications and future perspectives in tissue engineering. Further studies on this area and the provision of new production solutions will enhance the use of tissue engineering studies in "precision" or "personalized" medicine applications.

Types of Bioreactors Used in Tissue Engineering

The construction of completely functional three-dimensional (3D) artificial tissues and organs utilizing a biomaterials, cells, and signaling molecules is the ultimate objective of tissue engineering research. A dynamic culture that combines convection, perfusion, and diffusion is required to maintain 3D, clinically relevant sizes of tissue-engineered constructs (TECs) [121].

By stimulating cells with biochemical and physical regulatory signals, tissue engineering bioreactors can help the in vitro formation of new tissue by promoting cell proliferation, differentiation, and/or ECM production before in vivo implantation [48, 174]. Successful tissue engineering applications require the ability to maintain high cell populations over extended periods of culture without losing origin

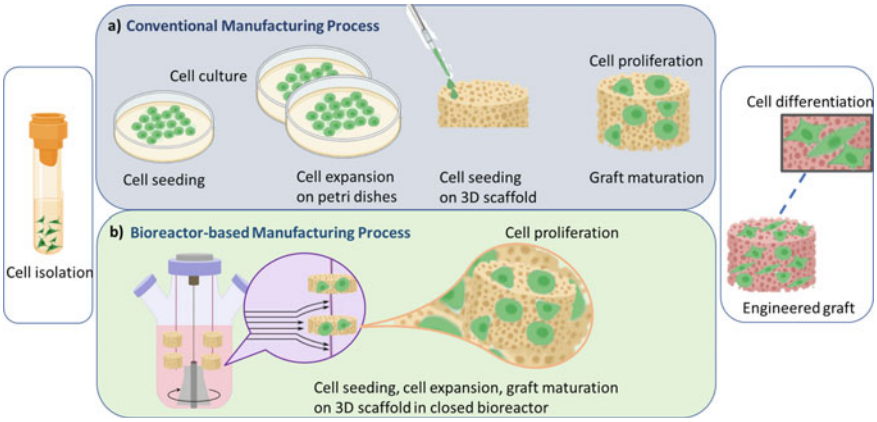

Fig. 9.1 Schematic representation of tissue formation approaches **a** conventional cell seeding **b** bioreactor-based. Created with Biorender (https://app.biorender.com/)

features, which is almost unattainable in static culture methods. Additionally, bioreactors offer a way to validate the functionality of the tissue engineering constructs before implantation under physiologically relevant loading conditions, as well as their preconditioning and maturement for some specific tissue engineering applications that require to tolerate significant mechanical loading immediately after implantation, such as heart valves and vascular tissues [47]. Bioreactors are also essential for tissue engineering applications on a realistic and larger scale because they enable aseptic procedures such as mounting, feeding, and sampling, while allowing control of environmental factors like nutrient provision, pH, pressure, oxygen level, waste removal, and temperature.

A variety of tissue engineering researches use conventional approaches (static seeding), which produce constructions with a thin tissue-like layer at the scaffold's base as a result of the cells' gravitational settling, as shown in Fig. 9.1. On the other hand, convective mixing (using spinner flasks) and convective flow (using flow perfusion) can enhance initial cell seeding and homogeneity, which in turn improves the tissue architecture [47]. Different tissue engineering applications employ a variety of bioreactors, and this chapter covers a range of bioreactors, such as spinner flasks, rotating walls, compression, perfusion, and microfluidic bioreactors (Fig. 9.2).

Spinner Flasks

The most straightforward bioreactor systems are spinner flasks which are cylindrical culture systems that have two arms that can be used to excise the stumps, and a magnetic rod attached to an impeller to circulate media and other culture components in the culture in a dynamic flow. In general, tissue engineering cultures in spinner

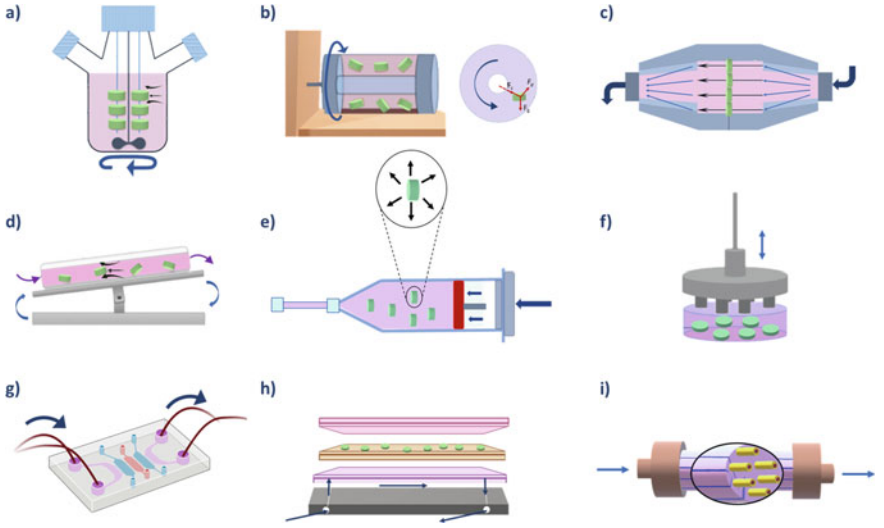

Fig. 9.2 A general scheme of bioreactor types; **a** spinner-flask bioreactor, **b** rotating-wall bioreactor, **c** perfusion bioreactor, **d** wave bioreactor, **e** hydrostatic pressure bioreactor, **f** compression bioreactor, **g** microfluidic bioreactor, **h** perfusion-parallel plate bioreactor and **i** perfusion hollow fiber bioreactor. Created with Biorender (https://app.biorender.com/)

flasks can be done in two ways; the cell/scaffold structures can be remained stationary in the flask or can be left to float freely in the spinner flask with the fluid flow. In the second option, the cells are exposed to less shear force and are less stressed [154]. These shear forces generally depend on the impeller's diameter size and rotational velocity. Therefore, in order to ensure the optimum cell growth, lower rotation rates are advised and implemented. The cell/scaffold constructs are mostly connected to the top of the flask with a needle, or the constructs are seeded onto microcarriers and immersed in the culture medium.

In spinner flasks, the uppermost section of the flask carries out gas exchange and medium oxygenation, while mixing tools like magnetic stirrers provide a well-mixed culture environment and increase the effectiveness of nutrition delivery and cell seeding in the scaffold. Standard stirring rates between 30 and 50 rpm promote dynamic medium mixing while minimizing harm to cells grown on the scaffolds [103]. The flow over the surface of the scaffolds creates vortices in the superficial pores of the scaffolds. Vortices are turbulence-related instabilities, and they may enhance fluid transport to the center of the scaffold, which increases cell viability and proliferation [58].

Spinner-flask technology was first used to allow biomass growth in cultures and has been used in tissue engineering studies for over 18 years. All culture parameters are well defined since it is a long-studied technology. The interactions between fluid dynamics and scaffold structure have become predictable using computational tools [49, 176]. Thus, spinner flasks are frequently encountered in applications involving

the expansion and engineering of various embryonic stem cell populations and the maturation of cells derived from adult tissues [150, 175]. Cartilage tissue engineering is one of the most common tissue types studied with spinner flasks. Since the cartilage tissue is not vascularized, basic bioreactor concepts like spinner flasks have high efficiency in cartilage regeneration [58]. Adipose Stem Cells (ASC) chondrogenic differentiation in spinner flasks has also been demonstrated, and this is linked to the creation of a spheroid culture that permits cell–cell contact. To create trachea transplants, rabbit mesenchymal stem cells (MSCs) were grown for four weeks in spinner flasks and macroaggregated on a PLGA (polylactic-glycolic acid) scaffold was demonstrated [89, 177]. In studies on bone tissue engineering, it was observed that the dynamic mixing environment of spinner flasks increased cell formation and osteogenic differentiation on the scaffold more than static culture environments [144].

The most important disadvantage of spinner flasks are the limited size of target tissue that can be cultured on the scaffold. Because the mass transfer (all the media and micronutrients required for cell culture) within the flask is not enough to provide homogeneous cell distribution along the scaffolds. Insufficient transfer causes uneven distribution and growth of cells on the scaffold. Increased mixing speed causes more shear stress on the peripherally located cells on the scaffold. Over time of culture, these cells seeded into the scaffolds cause more ECM and mineral deposition around the scaffolds increased amount of ECM and deposition of minerals around the scaffolds, resulting in a sharp apparent nutrient gradient and waste accumulation in the center of the scaffold, leading to cellular necrosis [41]. Also, inhomogeneous spread of the ECM around the scaffold severely affects the mechanical integrity of these structures. Rotating-wall vessel (RWV) bioreactors with a dynamic culture and low shear stress can be alternatives to spinner flasks, as discussed below.

Rotating Wall Vessel

Shear stress plays a crucial role in regulating the mechanical factors of tissue structures, nevertheless high shear stress leads to the formation of unwanted particules around the tissues. Hence, the need for bioreactors with low shear stress has arisen. The RVW bioreactor is the most common bioreactor in tissue engineering studies and reveals the benefit of low shear stress. It was first established by the National Aeronautics and Space Administration (NASA) to test simulated microgravity conditions, allowing cells to be grown in a microgravity environment [137].

The RWV consists of two concentric cylinders with a rotating outer cylinder and a fixed interior cylinder containing an oxygen-permeable membrane for gas exchange. The bioreactor is connected to a motorized drive system that enables it to rotate the system around the cylinder's axis at a slow, constant speed. The space between the two cylinders contains media and 3D cell/scaffold constructs. These constructs remain close to a "free-fall" state in a microgravity environment where drag forces,

centrifugal strength, and net gravitational forces are balanced and subjected to a dynamic laminar flow, as shown in Fig. 9.3 [162]. Because of this design, nutrient transmission increases, while shear stresses and turbulence are reduced. However, the low diffusion rate in the interior of the scaffold causes the inhomogeneous distribution of cells [19, 118]. Today, there are a variety of systems of the RWV, including Slow Turning Lateral Rotation Vessels (STLV), High Aspect Ratio Vessel (HARV), and Rotating Wall Perfused Vessel Systems (RWPV). Currently commercially available, STLVs are configured as an annular space between two concentric cylinders with an interior silicon gas exchange membrane, allowing greater control over culture parameters such as dissolved oxygen, pH, and temperature. On the other hand, although HARVs are similar to STLVs in general principle, they are more advanced in terms of gas exchange and culture rotation speed parameters [41]. RWPVs are designed to improve cell surface diffusion and mass transfer by convective flow under microgravity conditions [168].

Over the past century, RWV bioreactors are used in the culture of the retinal cell line to produce 3D-retina-like structures [39], temporomandibular joint disc [36], cartilage, and cardiac tissue engineering studies. It has been demonstrated that the

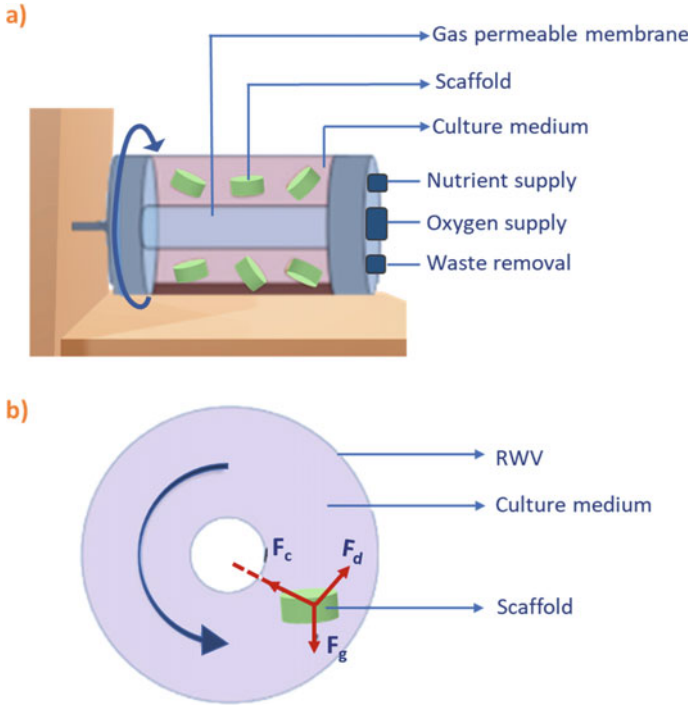

Fig. 9.3 **a** Schematic representation of a RWV bioreactor and **b** the centrifugal force (F_c), the gravity force (F_g) and the drag force (F_d) are balanced by the rotational motion of the bioreactor to keep the scaffold suspended. Created with Biorender (https://app.biorender.com/)

constructs produced by their culturing in RWV are structurally and functionally better than those produced using static or spinner flasks [23]. RWVs are also used in bone tissue engineering studies. Increased contact between cells and media enhances the proliferation and differentiation of osteoblastic cells due to better-controlled oxygen supply and less turbulence [111]. RWV-cultivated cells showed up regulations in markers indicating osteoclastic phenotypes compared to cells cultured in a traditional stationary culture, indicating that this environment can lead to higher bone resorption.

However, there are certain drawbacks associated with the use of RWVs. Cell proliferation is restricted to small scaffolds because sufficient transport of nutrients cannot be assured into the inner part of scaffolds [58]. In some studies, scaffolds have been reported to collide with the walls of the rotating vessel chamber during culture, causing cell damage and impairing the attachment of cells and matrix deposition on the scaffolds. When newly developed scaffolds with a density lower than water (like PLGA) are used, higher levels of ALP and calcium are observed in osteoblasts compared to static culture [24]. Although these findings are encouraging, the lack of effectiveness shown in RWV systems has led academics to look at other dynamic culture systems.

Perfusion Systems

A perfusion system generally includes a medium reservoir, a pump, and a conjunction system with columns, chambers, or cartridges that engage the cell/scaffold constructions, which can be used to improve cell growth [175]. In order to allow the medium to flow through the pores of the scaffold instead of around it, scaffolds are fitted tightly to the bioreactor cartridges. Through improved nutrition delivery to the interior of the scaffold and providing mechanical stimulation from liquid shear, medium flow through the scaffold porosity promotes cell differentiation (Fig. 9.4) [102].

Because perfusion bioreactors use a pressure gradient, they provide more even cell distribution and tissue-specific protein expression compared to constructs stimulated in a spin flask bioreactor. Since the mechanical loading regime most nearly mimics the condition that occurs in vivo, perfusion bioreactors are most widely employed for bone tissue engineering applications. Other cell types, including as MSCs, chondrocytes, keratinocytes, hepatocytes, and cardiomyocytes were successfully cultivated in perfusion systems for the creation of a TECs [52, 122, 131, 140]. Also, they enable the development of cell and scaffold constructions that have been computationally planned and printed into whole tissues [43]. Decellularization, developing technology to remove cells from original tissues' ECM in order to create 3D organ/tissue scaffolds for TE, is another use for perfusion bioreactors. Several tissues and organs have been effectively decellularized with the use of perfusion bioreactors [160].

With the perfusion system that provides continuous media flow, harmful metabolites can be eliminated, and mass transfer is enhanced growth factors and nutrients are continuously supplied. There are different configuration types of these bioreactors;

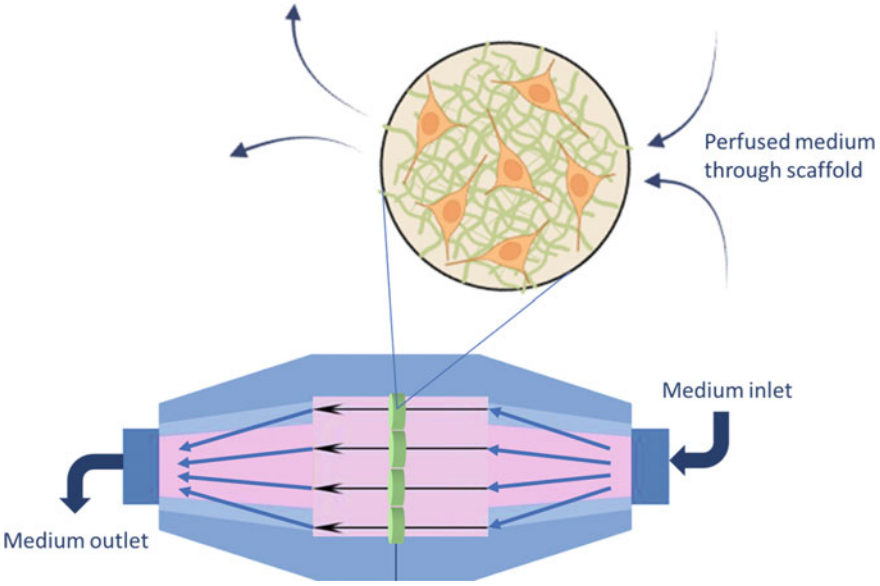

Fig. 9.4 General schematic representation of a perfusion bioreactor. Created with Biorender (https://app.biorender.com/)

like parallel-plate bioreactors, hollow-fiber bioreactors (HFBs), fixed (packed)-bed bioreactors, and fluidized-bed bioreactors [111].

Parallel plate bioreactors are composed of polystyrene plates and each plate has two compartments separated by a gas-permeable/liquid-impermeable membrane. The bottom compartment from those compartments contains the cells adhered as a monolayer to the surface, and is filled with the culture medium, whereas the upper compartment is filled with a mixture of gases. Cell mechanotransduction research with Parallel-Plate bioreactors usually makes use of well-defined shear stress resulting from the laminar flow that simulates intracellular environments [16, 170]. In the early years of tissue engineering, although a few studies were using this type of bioreactors, such as skin tissue and bone tissue studies, it is not widely used today.

HFBs offer high surface area-to-volume ratios (100–200 cm^2/L) and give cells a 3D environment for cellular attachment and proliferation with a low level of shear stress [120]. The system is composed of hair-like hollow fibers made of cellulosic, polysulfone, polypropylene, or polyethylene materials within a tubular cartridge that has inlet and outlet ports for flow around or inside the fibers. The cells are cultured on the interior or exterior surfaces of the hollow fibers, which are semipermeable tubular membranes with pores that range in size from 10 kDa to 0.3 m [172]. These pores of hollow fiber membranes also prevent the passage of unwanted molecules. Hollow fiber bioreactors are used for tissue engineering studies of tubular-shaped tissues such as blood vessels, intestines, and urinary organs, as they mimic the natural capillary

system, and facilitate the generation of heterogeneous tissues with these bioreactors. Apart from that, they are well-suited for cultivating cells with high metabolic activity, such as hepatocytes, as they provide a high mass transfer rate [65, 77]. Apart from those a HFB would be less suitable if the research plan involves harvesting cells, due to the difficulty of harvesting cells that adhere to fibers.

Fixed or packed-bed bioreactors (PBRs) are small and compact systems contributing high productivity. They consist of a tank that contains the culture medium and an immobilized matrix of particles compactly packed enclosed in a column. Particles in the immobilized matrix can be composed of porous ceramic beads, macroporous microcarriers, porous glass beads, polyester discs, glass fibers, hydrogels, and alginate beads and allow cells to grow within or on them, while the tank provides oxygen and nutrients to cells through the bed. In these bioreactor configurations, both units are coupled via a circulation loop through which an oxygen-enriched culture medium is perfused through the fixed-bed [38, 111]. Thus, while the cells (adherent or non-adherent) are retained in the fixed-bed, fresh medium is supplied to the cells by perfusion, and toxic metabolic products are removed from the cells. The main and only difference between Fixed-Bed bioreactors and Fluidized-Bed bioreactors is that the particles in which cell growth is achieved are either packed (fixed) or floating (fluidized). In a fluidized bed bioreactor, culture media is continuously pumped upwards to a group of particles, cells, or immobilized cells, causing them to be suspended and behave as though they were fluid. Due to its uniform bed expansion behavior, superior mass transfer qualities, low shear stress, and straightforward scale-up, FBB have grown in popularity in biotechnology operations [33].

Although perfusion systems manage the problems related to other bioreactor systems and static culture, and using these bioreactors and their derivatives seems promising in clinical scenarios, these types of bioreactors have some limitations. Because of improper connections within the system, these systems are vulnerable to contamination and leakage. Also, significant optimization of process parameters and scaffold designs are required for maximizing the yield of the culture process.

Pulsatile Perfusion Bioreactors

Pulsatile perfusion bioreactors have been developed to mimic the pulsatile physical forces and in vitro cardiovascular conditions that vascular cells are exposed to during vasculogenesis. The system, first developed by Niklason et al., to mimic in vitro cardiovascular conditions, provides intraluminal pulsatile flow to four reactors, each of which contains one construct. Thanks to the pump in the perfusion pulsatile system, it can be operated at pulse rates at defined beats/minute intervals by applying pressure at variable stroke volumes [110].

Although the general operation of pulsatile bioreactors is essentially the same, minor modifications in reactor design might result in significantly varied hemodynamic conditions and, thus, varied outcomes for preconditioned cell-seeded heart

valves. In the literature, there are several studies conducted by investigators to engineer pulsatile bioreactors to condition intact tissue-engineered heart valves. The first compact heart valve bioreactor, created by Hoerstrup et al., is composed of an air chamber, a media chamber, and a perfusion chamber that holds the heart valve. A reciprocating pneumatic diaphragm located between the air and media chambers in the reactor provides pulsatile flow [62]. On the other hand, Weston & Yoganathan engineered a tubular pulsatile flow bioreactor to evaluate compartmentalized leaflets that were sutured into a tubular structure. A heat exchanger and a gas infusion filter were employed in this bioreactor to enable the system to operate physiologically outside of the incubator. Pumps were utilized to create a constant and pulsatile flow throughout the closed loop system [167]. The bioreactor designed by Lichtenberg et al. includes a pulsatile pump, heart valve reservoir, media reservoir, and oxygenation/compliance chamber. This closed loop system created allows direct control of the flow rate thanks to a pulsatile pump, while monitoring the conditions inside the bioreactor with flow, pressure, and temperature transducers [86].

By virtue of perfusion bioreactors' ability to simulate the physiological and chemical conditions of living tissue, it has been possible to research in vitro cellular responses and develop better, and more effective tissues. Tissue-engineered heart valves still need to be thoroughly studied, and several significant problems need to be overcome before they can be used in clinical practices. There is no established conditioning technique for pulsatile perfusion bioreactors because there are so few clinical studies on the disease. Future research should pinpoint the conditions of these bioreactors that will promote clinical success.

Rocker Platforms—Wave Bioreactors

Wave bioreactors are disposable single-use bioreactors that typically consist of a transparent flexible polymer bag. Wave-induced agitation is achieved by placing the bioreactor bag on a rocking platform. For this reason, they are also referred to in the literature as "Rocker platforms" (Fig. 9.5).

The rocking platform can be either an open system that can be kept in an incubator or a closed system with a controlled environment. To accomplish the essential gas transfer through the headspace of the bag and culture homogenization, depending on the needs of the cultured constructs, the geometry, filling level, rocking angle, and rocking velocity of the wave bioreactor, as well as the viscosity of the medium, must be adjusted [20]. Due of the minimal shear stress generated by rocking without mechanical mixing, mass transfer is increased. Additionally, the technology is appropriate for sensitive cells like stem cells since it offers bubble-free aeration. These systems have a reduced risk of contamination since they use disposable bioreactor bags. As with all closed systems, it allows monitoring and control of temperature, pH and DO [8].

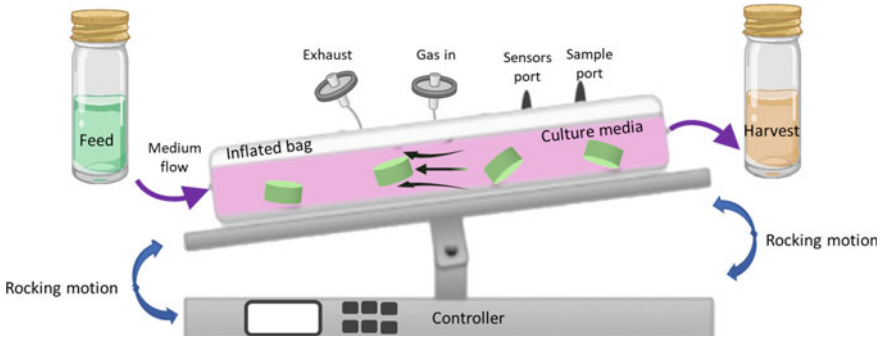

Fig. 9.5 Schematic drawing of a wave bioreactor. Created with Biorender (https://app.biorender.com/)

The initial version of the wave bioreactor was originally released on the market as a disposable replacement for stainless steel bioreactors in the 1990s. The original technology which was created by Singh consisted of a disposable plastic bag positioned on a motorized platform that was controlled outside the incubator and contained culture media and cell inoculum [145]. Without affecting the fluid shear or gas bubbles, the platform performs a rocking motion enabling good mixing and gas transmission. Unified sensor technology and control software not found in the early versions have been added to new designs to enhance automation, reliability, and repeatability [146].

Wave bioreactor can perform in batch, fed-batch, repeated fed-batch, and continuous perfusion modes, and it has auxiliary ports for connecting culture media bags for perfusion. When the bioreactor is performed continuously, the harvest bag and feed bag are integrated to allow continuous supply of fresh medium and removal of the waste medium [81]. A fed-batch system is the most favored wave bioreactor process since its ability ease of use and for eliminating the possibility of substrate/product inhibition seen in batch systems [40]. It has been shown in studies in the literature that scale-up and system automation are facilitated by using the batch feed process, with volumes up to 500 L [156]. It has been demonstrated in propagation cultures of mammalian cells, such as neutrophils from hematopoietic stem cells [156], embryonic feline lung fibroblasts [67], and T cells [57], that waves produced by shaking suspend cells/aggregates, thereby increasing mass transfer [96, 153].

Except for cell proliferation, where static bags are widely common, wave bioreactors are practically insignificant for functional tissue production. Based on the literature, there was only one official report of 3D tissue production in a disposable wave bioreactor. In the study conducted by Halberstadt et al., the production of human dermal replacement was achieved in a system consisting of 16 wave-bag reactors operating in perfusion mode, a 16-channel peristaltic pump, reservoir bag and waste bag. Each bag consists of a biodegradable free-floating 3D mesh scaffold to provide the necessary template for cell growth and skin tissue development. Tissues obtained after 22 days of culture with this system were histologically comparable to

tissues produced in both continuously perfused and fed static culture bags of decorin, collagen type I, and fibronectin deposition [56]. Since the outcomes were promising, the perfusion bioreactor was adapted to produce obtainable through commercial channels tissue-engineered molting Dermagraft® in disposable bags [126]. Wave bioreactors do not require being sterilized, and scaling them up is relatively simple, but each research needs to optimize the rotating speed, angle, and bag fill level.

Microfluidic Bioreactors

Microfluidic bioreactors, also known as perfusion microbioreactors, biochips, or cell chips are a miniaturized version of conventional bioreactors with at least one perfused channel with a size in the micrometer range (Fig. 9.6). Similar to macroscale bioreactor systems, microfluidic bioreactor systems integrate monitoring and control components. They were developed to address a number of issues that were present in conventional systems, including the high consumption of growth medium and components like growth factors, limited compliance with high-throughput screening, challenges in controlling parameters and the microenvironment, elevated manufacturing expenses, difficulties with live-cell analysis and imaging, and the inadequate supply tissues with oxygen and nutrients [117]. Through channels, microliter quantities of fluid can be delivered to cells to properly evaluate the impacts of various doses of growth factors or pharmacological drugs.

The requirement to cultivate cells under shear stress has led to the development of microfluidic bioreactors. Unless specific features like actuators or surface modifications are included, the flow regimes within the microfluidic system are always laminar due to the small geometry of the channel [84, 178]. In addition to ensuring a constant flow of nutrients and the elimination of waste materials, the laminar flow regime also applies precise mechanical stress to the cells grown inside the channels. The

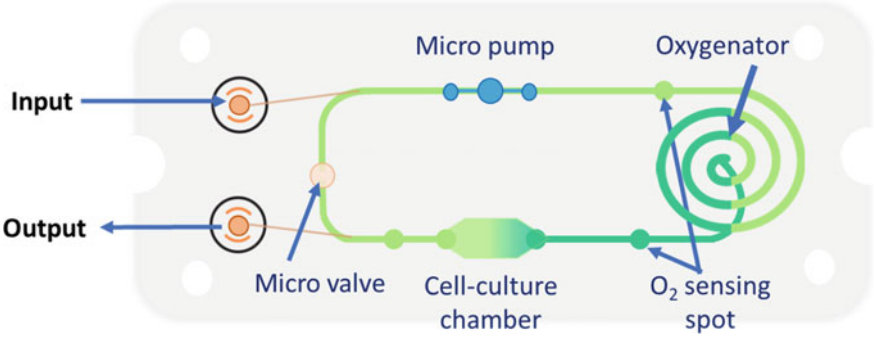

Fig. 9.6 A scheme of a microfluidic bioreactor. Created with Biorender (https://app.biorender.com/)

shear stress sensitivity of stem cells makes microfluidics an advantageous method for exploring stem cell differentiation caused by mechanical stimulation [44].

Microfluidic systems are fundamentally insufficient for growing cells or tissue-engineered products where large cell populations or complex structures are required due to their miniature geometry. The small size, however, has a number of benefits, including quick reaction times and minimum reagent use. Microfluidic technology also lends itself to automation and sensor integration. These characteristics make microfluidics an ideal technology for developing testing devices for toxicity and drug screening as well as for fundamental research [44]. Improvements to these systems have made it feasible to utilize multicellular aggregates, microspheres, and cell encapsulation in high-density 3D cell culture to more accurately mimic the interactions between native tissue cells than is possible in 2D culture. However, it is challenging to investigate how pharmacological substances affect the complex processes of tissues like the heart or lung in microfluidic bioreactors. As a result, most recent lab-on-a-chip bioreactor designs combine physiological factors like airflow and mechanical stimulation that simulate respiration or integrated vascularization and direct blood flow with contractile heart cells [9, 180].

Microfluidic bioreactors have several applications in a broad range of fields, including biochemical analysis, drug development, environmental monitoring, DNA and protein separation, and analysis. Aside from these, it is employed in subsidiary branches of cell biology including adhesion, spreading, proliferation, and differentiation, as well as in monitoring toxicity, counting, and sorting cells, and signaling pathways [2, 17, 78, 88, 141]. With such a diverse set of applications, it is possible to examine single cells, cell populations, tissues, and even complete organs like the skin-on-a-chip, vasculature-on-a-chip, bone-on-a-chip, brain-on-a-chip, kidney-on-a-chip, muscle-on-a-chip, heart-on-a-chip, lung-on-a-chip, liver-on-a-chip, gut-on-a-chip, multiorgan-on-a-chip, or tumor-on-a-chip in vitro [6, 53, 59, 74, 76, 106, 112, 143, 182]. Accordingly, the use of microfluidic bioreactors for cell studies is spreading quickly, with novel designs and microenvironments continually arising as a result of the incorporation of various materials, processing methods, and functional components.

Particularly in the areas of drug screening, tissue engineering, and organ transplantation, microfluidic devices have the potential to have a substantial influence on a wide range of biochemical applications. In contrast to macroscale bioreactor systems, tissue culture in microscale devices offers a more comprehensive model for analyzing the cellular response to stimuli and the ability to regenerate cellular microenvironments [64, 68, 166]. It has taken a significant amount of effort to create appropriate microfluidic systems that allow for the quantitative control of cell culture parameters for tissue growth. For instance, spatial and temporal gradients that control cell proliferation, migration, and differentiation are essential to the formation of tissues. Long-term cell culture, live-cell imaging of individual cells, and cell tracking to ascertain destiny are all possible with microfluidic technology [128]. Systems based on smart phone designed for internal environment and hybrid materials monitorization that enable point-to-point cell manipulation inside the bioreactor are some of the most recent developments in lab-on-a-chip technology [27]. The development of

increasingly sophisticated human-on-a-chip systems that will investigate the effects of pharmaceuticals on various organ systems both directly and indirectly is predicted by current developments.

Strain Bioreactors, Compression Bioreactors and Hydrostatic Pressure Bioreactors

Strain bioreactors have been designed to directly apply mechanical stress to various mechanically responsive tissue cultures, such as bone, ligament, tendon, cartilage, and cardiovascular tissues [34, 50, 134]. Through a strain bioreactor, a direct mechanical strain may be imposed in the ways of stretching, compression, and bending. In strain bioreactors, the clamps attached to the scaffold are often employed to transfer the tensile force for 3D constructions, and linear actuators with digital control are used to manage tension [107]. To minimize structural damage during loading, the design of clamps must be altered based on the intended use. Various types of clamps have been developed to optimize scaffold assembly because it is pivotal that they do not cause cracking or tearing in the scaffolding they are mounted on. For example, spiral grips and attachment hooks are used for thicker structures, while grip pins and standard clamps are used for thinner structures [18, 99]. Alignment of cells at a 90-degree angle to the stretch direction is known to be induced by cyclic stretching and may produce homology to the target native tissue [134, 159]. According to several studies, the degree of alignment relies on the waveform of the stretch, frequency, and magnitude. In order to better simulate the physiological circumstances in tissues like the peritoneum, skin, and aortic valves, stretching can also be biaxial or equiaxial [71, 83].

In engineering studies of tissues exposed to compression in the natural environments like cartilage in the knee joints and bones, a compression bioreactor is adopted, which can provide both static and dynamic loading. Only the manner by which the force is applied to the structure differentiates these bioreactors apart from strain bioreactors. A standard basic compression bioreactor is made up of a motor that can apply linear motion and a control system that allows the operator to choose between various magnitudes and frequencies [63].

The bioreactor offers a regulated environment to establish a compression load bioreactor, it is essential to identify the compression type (dynamic or static compression) and determine its strain amplitude, frequency, and duration, in order to build a compression load bioreactor. Such bioreactors may be designed to offer both dynamic and static loading, allowing them to be adjusted for various application types [121]. Dynamic loading, which simulates more physiological loading, showed improved results than many other stimuli, despite the fact that static loading, which only allows for limited mass transport, has a negative impact on cartilage growth. Further studies have demonstrated the stimulatory effects of compressive strain on the scaffold elastic

modulus, sulfated glycosaminoglycan (GAG), and hydroxyproline concentration in cartilage tissue engineering [31, 42, 101].

Another method of providing mechanical stimulation to structures made of tissue engineering is the use of hydrostatic pressure bioreactors. By covering a monolayer of cells grown in a petri dish with culture media and putting them in a pressure chamber where a gas phase works on both sides of the dish, hydrostatic pressure may be transmitted to the cells. Scaffolds are often statically cultured in cartilage tissue engineering research before being moved to a hydrostatic chamber for loading for a prescribed time frame [25, 61, 181]. The essential components of a hydrostatic pressure bioreactor are a chamber with the capacity to resist the applied pressures, pumps or pistons to apply that pressure, filters for ventilation, and non-return valves. As an example, an actuator-controlled piston may be used to pressurize a pressure chamber that is filled with media. The plunger can pressurize through an impermeable membrane to maintain sterility while preventing direct contact between the plunger and the culture media. A water-filled pressure chamber that uses a variable back pressure valve and an actuator to pressurize a media-filled chamber via an impermeable film is one variation of this idea [127, 165]. The hydrostatic pressure application's ideal magnitude, frequency, and period have not yet been determined. Dynamic hydrostatic pressure was described as preferable to static hydrostatic pressure in terms of how effectively chondrocytes proliferated in a monolayer [181].

To determine the most effective magnitude, frequency, and duration of applying strain, compression, or hydrostatic pressure using bioreactors, a case-specific approach is necessary. This approach must consider factors such as scaffold type and shape, as well as changes in cell number, porosity, and elastic moduli resulting from deposited ECM during the culture period. While it is possible to design and construct bioreactors that can apply various types and magnitudes of strain, compression, or hydrostatic pressure, a case-specific approach is needed (Fig. 9.7).

Combined Systems

In contrast to the basic loading circumstances caused by the different kinds of bioreactors discussed in this chapter, physiological loading conditions in the body are significantly more complicated [8]. As given in Table 9.1, the applications can be varied according to advantages and disadvantages of bioreactors. Combined systems are used to overcome their disadvantages or increase their advantages. Combinations of several bioreactor types can be employed to simulate the in vivo environment in vitro more effectively, fulfill the loading requirements for tissue-specific applications, and more accurately model the original tissue microenvironment. Stretching, compression, or perfusion cycling on HP bioreactors is the most popular use for combination bioreactors. Nutrient exchange is made possible in these various bioreactors designed for engineering certain tissues by perfusion, while stimulation is made possible through various mechanical stimuli [13, 165].

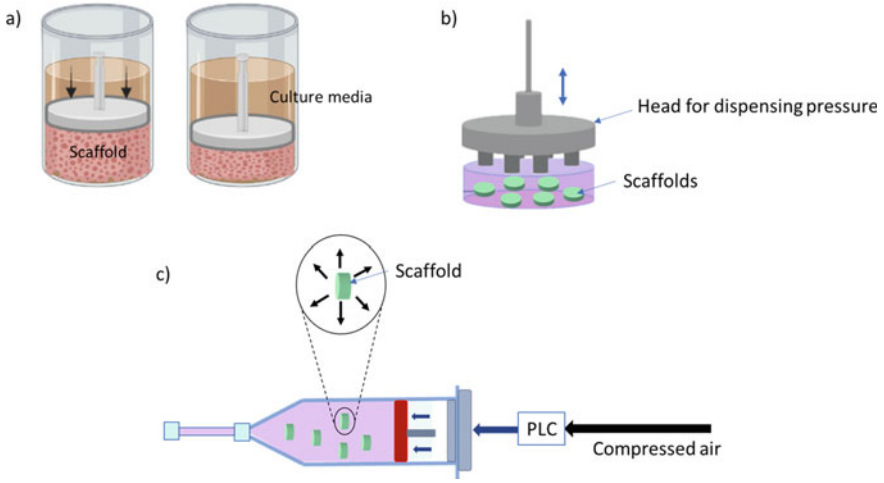

Fig. 9.7 **a** Strain bioreactor **b** compression bioreactor and **c** hydrostatic pressure bioreactor. Created with Biorender (https://app.biorender.com/)

A perfusion-loop tension and vibration bioreactor made for vocal cord tissue engineering that may simulate airflow-induced stimulation is one of the most well-known instances of a combination bioreactor. The bioreactor, which consisted of two synthetic vocal fold replicas in a silicon body, used airflow-induced self-oscillations, which have been demonstrated to create mechanical loading and contact forces that replicate human phonation. It was determined at the end of the study that the phono-mimetic bioreactor supports ECM protein production and cell survival as projected [82, 157]. In research by Dermenoudis and Missirlis to simulate blood vessels, four mechanical stimuli have been established and developed: (1) normal and (2) blood pressure-related environmental stress, (3) shear stress from blood flow, and (4) individually controlled rotation-induced gravitational field. Rotation was found to be the most complicated stimulus in the study,when used alone, it causes the cells' polarity axes to shift frequently, and when coupled with other stimuli, it prevents elongation without changing the orientation profile [35].

In a study, scaffold-free cartilage constructions produced by porcine chondrocytes were cultivated under static and compression conditions to investigate the effects of perfusion and cyclic compression. GAG content was discovered to be considerably higher in the mechanically loaded group than in the statically loaded group and native tissue at the conclusion of the research [161].

Although combined systems provide a superior degree of tissue, size, and scaffold specific in vivo stimulation, they also add complexity and offer less control over testing parameters. Biological reactions to combined loading are typically challenging to predict and certainly do not total to the sum of the individual effects. There are multiple interactions among various cellular components, making it more

Table 9.1 Bioreactor types, advantages, disadvantages and applications in tissue engineering

Type of bioreactor	Type of stimulation	Advantages	Disadvantages	Applications
Spinner Flask	Shear stress	Exposure of the scaffold to shear results in enhanced cell proliferation rate, matrix deposition, and expression of proteins specific to the phenotype	Inhomogeneous distribution of the ECM Limited size and mass transfer	Musculoskeletal tissue engineering [1] Cartilage tissue engineering [58]
Strain	Compression Tension Bending Torsion Pressure	Ability to simulate physiological loading conditions Enhanced cell growth rate, matrix maturation, and expression of a variety of phenotype specific proteins	Risk of construct damage caused by the mounting of scaffolds and the application of direct strains Limited mass transfer	Cardiovascular tissue engineering [4] Musculoskeletal tissue engineering [34, 50, 134]
Rotating wall vessel	Low shear stress, reduced gravity conditions	Protection of cells from exorbitant shear stress and turbulence Simulation of microgravity	Time-consuming for optimization of the culture conditions Cells damage caused from scaffold colliding to the bioreactor wall Limited mass transfer Limited cell proliferation	Musculoskeletal tissue engineering [1] Cardiovascular tissue engineering [4] Ocular tissue engineering [39]

(continued)

difficult to optimize the right timing, quantity, and frequency of the parameters as the number of stimulation factors rises.

Future Perspectives on Tissue Engineering Bioreactors

Since the bioreactors have the potential to increase process efficiency, particularly for the clinical application of tissue engineering constructions, they are quickly becoming an essential component of tissue engineering research. Improved mass

Table 9.1 (continued)

Type of bioreactor	Type of stimulation	Advantages	Disadvantages	Applications
Perfusion	Shear stress	Better cell distribution and tissue-specific protein expression Can be automated Mimicking in vivo physiological environment of the tissue Limited turbulence	Appropriate only for scaffolds that are both mechanically strong and highly porous Vulnerable to contamination and leakage Optimization of flow rates is vital High rates of flow induced shear can cause cell and membrane disruption	Musculoskeletal tissue engineering [1] Cardiovascular tissue engineering [4] Kidney tissue engineering [147] Lung tissue engineering [116]
Perfusion-hollow fiber	Low shear stress	Limited contamination Increased surface to volume ratio	Not suitable for cell imaging Difficult to harvest cells expensive commercial HFBs	Urinary tissue engineering [77]
Perfusion-parallel-plate bioreactor	Shear stress	Well-defined shear stress Simulations of intracellular environments Easy to manufacture Inexpensive	Difficult to employ for 3D constructs	Skin tissue engineering [170]
Pulsatile perfusion bioreactor	Pulsatile physical forces Low shear stress	Simulation the physiological and chemical conditions of living tissue	Requirement for maintaining medium reservoir's temperature a little higher than desired temperature for the valve chamber due to heat loss	GI system tissue engineering [70] Cardiovascular tissue engineering [4] Kidney tissue engineering [119]

(continued)

Table 9.1 (continued)

Type of bioreactor	Type of stimulation	Advantages	Disadvantages	Applications
Microfluidic	Shear stress Tension Compression Pressure	Completely controlled mechanical stimuli Reducing the use of culture components Enabling high throughput screening and lowering production costs	Diffuculties for obtaining Organ-size products Requirement for adjustment for each study due to the decreased scale	GI system tissue engineering [173] Lung tissue engineering [80] Organ-on-chip [182]
Wave	Shear stress	Low shear stress Suitability for sensitive cells due to bubble-free system Easy to scale-up	The requirement to optimize the rocking rate, velocity, angle, and bag filling level for each study Limited mass transport High cost	Dermal tissue engineering [56]
Combined	Shear stress Compression Tension Bending Torsion Pressure Electromagnetic	Ability to apply different kinds of stimuli simultaneously	Requires a higher level of expertise Difficult optimization due to the increased number of parameters	

transfer, tightly regulated culture conditions, physiologically suitable stimuli, continuous medium supply, reduction of process steps, automated sampling for quality control, and standardization may all be offered by tissue engineering bioreactors [8].

Since poor cell viability caused by a lack of vascularization has been the rate-limiting factor in the efficient implementation of tissue engineering constructions in clinics, improved mass transfer is by far the main goal of employing tissue engineering bioreactors. With the help of porous scaffolds and precise perfusion, bioreactors provide appropriate oxygen, nutritional, and biosignal availability to the interior of tissue engineering constructions while supporting the development of the tissue in bigger dimensions than statically diffusible 100–200 m layers. When designing a new generation of modern bioreactors to simulate the physiological tissue microenvironment involving biochemical, biophysical, mechanical, and electromechanical parameters more accurately, the following factors should be taken into account.

- A sufficient environment for in vivo vascularization should be supplied after implantation, as in vitro vascularization of tissues is a priority in tissue engineering.

- The inflammatory environment must be considered as a crucial element of the mammalian host tissue response for a biomimetic approach.
- Continuous monitoring of the bioreactor environment and tissue growth using advanced imaging and sensing methods to track cell fate and tissue development in the intricate 3D environment [20].

The optimum automation of bioreactor control requires real-time and non-destructive evaluation of tissue and organ regeneration. To report inputs of environmental signals (such as mechanical actuation, oxygenation, or transfer of biological components), imaging and sensor data can be employed in a feedback loop. The advent of biotechnology and nanotechnology has altered how we perceive medicine and what we expect from healthcare systems. Future medical procedures, including tissue engineering, must be customized to each patient's needs in order to practice "precision" or "personalized" medicine, which is becoming more and more significant. Personalization of tissue engineering constructions would need bioreactors created specifically to meet the requirements of the individual patient. The use of patient-specific culture medium with specified loading conditions, patient-derived cells, and scaffolds that have been (bio)printed in the size and form of the desired defect is nonfiction.

Critical Parameters in Bioreactor Design

Factors to be ensured during three-dimensional tissue fabrication in bioreactors are maintaining a uniform cell concentration when seeding cells on the scaffold, control of microenvironmental parameters (temperature, pH, pressure, DO, metabolites, shear stress, and agitation), and aseptic parameters. In order to deliver nutrients and oxygen to cells and regulate the elimination of metabolic waste from the environment, a bioreactor offers a biomechanical and biochemical environment [8].

One of the most critical parameters during cell seeding on scaffolds in bioreactors is mass transfer. During long-term culture, cell viability needs to be maintained in the interior of the construct after cells are seeded on porous scaffolds. In order to maintain cell viability, nutrients, oxygen, and regulatory molecules must be transferred efficiently from the culture medium to tissue surfaces and inner cells of the tissue structure is required. At the same time, CO_2 and metabolites from the tissue's cells must be transferred to the bulk medium [87] (Fig. 9.8).

Mass transfer between a moving fluid and a surface is called convective mass transfer. In a bioreactor, the external mass transfer rate depends on hydrodynamic conditions. In a system with concentration difference, mass transfer occurs either by molecular (diffusion) or by convection. The internal mass transfer rate depends on the tissue structure, size and porosity of the scaffold, the diffusion rate of the cell and molecules from the biomaterial, and utilizing of both convection and diffusion mechanisms. According to the design of the scaffold, an efficient mass transfer can

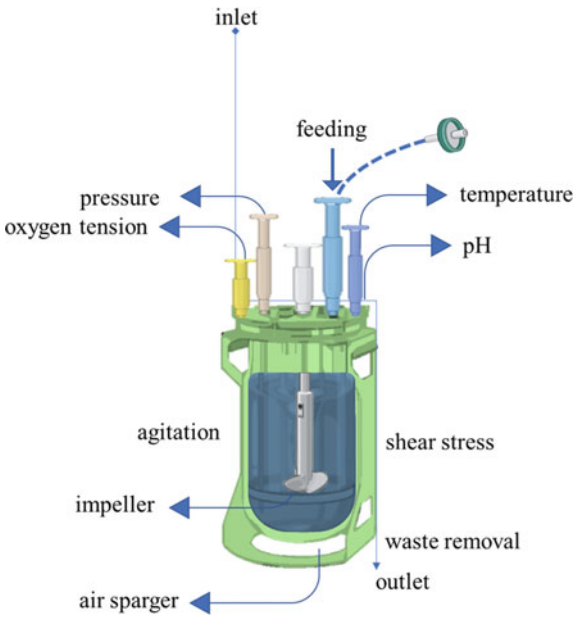

Fig. 9.8 Design parameters of bioreactors. Created with Biorender (https://app.biorender.com/)

be achieved by determining the flow direction and flow rate of the mass transfer [28, 87].

Every organism has an optimum temperature value at which it performs its metabolic activities. The optimal physiological temperature at which most mammalian cells continue their vital functions is 37 °C. At a temperature above 38 °C, it has a rapidly lethal effect on cell viability. At lower temperatures, cell metabolism slows down. In this case, the temperature of the culture medium in the bioreactor must be kept uniformly constant. The bioreactor's water jacket, temperature control unit, and temperature sensor work together to regulate this. The jacket that surrounds the bioreactor tank is a water-containing system. The jacket in contact with the bioreactor ensures that the temperature of the culture medium is balanced and remains constant. The temperature control unit receives a signal from the temperature sensor, which subsequently adjusts the temperature based on the culture medium's actual temperature. Water or any other heat transfer fluid circulating in the jacket around the bioreactor tank is heated or cooled by the temperature control device. As a result, the bioreactor's culture medium temperature is balanced by comparing it to the jacket's temperature [10, 28].

Hydrogen ion concentration (pH) significantly affects the metabolic activities and growth of organisms. Every organism has an optimum pH range in which it shows maximum activity. The optimal physiological pH required for mammalian cells to continue their vital functions is in the range of 7.0–7.4. As a result of cellular metabolisms converting glucose to lactate, the pH value of the culture medium decreases due to the production of CO_2 and water. For this reason, the culture medium becomes more acidic when no treatment is done during the culture. A bicarbonate

buffer is used to keep the pH value of the culture medium at an optimal value. The pH is balanced by changing the amount of bicarbonate used in the culture. In addition, CO_2 can be added to the culture medium with a sparger to decrease the pH value, or air can be added to the sparger to increase the pH value. When the pH value of the culture medium in the bioreactor needs to be increased, a basic solution such as NaOH or Na_2CO_3 can be added. As a result of adding air, CO_2 or basic solution to the bioreactor, the pH probe in the bioreactor measures the pH value of the environment and is automatically brought to the optimal value by the controller [28, 87].

Oxygen, which is one of the components that must be present in the culture medium, is rapidly consumed by the respiration of the cell. Oxygen, which has low water solubility, must be continuously supplied to the culture medium. In order to avoid oxygen limitation in the culture medium, it is aimed that the OTR be greater than the oxygen utilization rate. For this reason, air, air and O_2 mixture or pure O_2 can be added to the bioreactor by air sparger to ensure the continuation of the culture medium. Continuous gas entry into the bioreactor is provided by the sparging air, which is usually located under the impeller. With the sparging air under the impeller, the circulation of the gas given to the bioreactor is ensured. Another important parameter in the culture medium is the DO level. Most mammalian cell cultures are able to continue their metabolic activity at DO of around 20–50% of the saturation with air. The DO level in the environment is detected by a sensor. The addition of air/O_2 to the bioreactor is managed by the controller according to the difference between the value measured by the DO sensor immersed in the culture and the desired value. When there is a higher level of DO in the medium than the desired value, nitrogen can be added to the bioreactor via the sparger to remove the oxygen from the culture medium. In another widely used method, cells are allowed to consume oxygen up to a certain point. As a result of O_2 consumption, air, O_2 or air/O_2 mixture is given with the sparger to raise the DO level again below the determined value. Allowing the cells to consume oxygen until the target value is reached is an alternative and more typical common approach. When a setpoint is not reached by the process value, a mixture of air and O_2 is added to raise the process value back to the setpoint for DO [105, 123, 135].

Oxygen transfer in the culture medium is important because of the poor solubility of oxygen in the culture medium. A balance must be maintained between the oxygen supplied to the cells and the oxygen consumed by the cells. Therefore, another critical parameter during the design of the bioreactor is the oxygen tension setting. In tissue engineering applications, the oxygen requirement of the cells in the culture medium varies according to the phases in the growth curve. During the initial expansion phase, the overall oxygen demand increases as the cell density increases with time. In the next process, the cells go from the state of reproduction to the state of differentiation, and in the case of differentiation, the oxygen requirement of the cells that use less oxygen decreases [126].

The culture in a reactor can be aerated by aeration, direct scattering, indirect and/or membrane aeration (diffusion), medium perfusion, this helps to increase the atmospheric pressure and the partial pressure of oxygen. In a bioreactor, DO can be transported by global mass transfer, internal mass transfer, or external mass transfer.

The rate at which oxygen is given to the environment at the gas–liquid interface is constrained by how soluble oxygen is in water. The flow area of the vessel and the net consumption or production rate determine the oxygen concentration distributions in the culture medium for global mass transfer. The equilibrium between the oxygen provided to the environment, known as the OTR, and the oxygen consumed by cells, known as the oxygen absorption rate, determines the oxygen concentration in the environment. The liquid phase mass transfer coefficient (kLa) and productivity are significantly impacted by the OTR ratio [93, 105].

It is acknowledged that mechanical stimulation—such as pressure, tension, hydrodynamic pressure, and fluid flow—is crucial for the maturation of organs in the bioreactor. Mechanical interactions between the culture medium and the scaffold during tissue growth determine whether cells form cell clumps or disperse on the scaffold. Determining optimal physical parameters is complex due to the diversity of cell types, scaffolds, forces, regimens applied, and culture medium available. An impeller system is used to ensure a homogeneous distribution of the culture medium in the bioreactor and for air circulation [105]. The agitation system basically consists of a rotor, a drive mechanism (magnetic or direct) and a motor. The bioreactor can be powered to achieve effective mixing and a uniform distribution of temperature, DO, and pH in the culture medium. The spreader's design, the impeller's type, size, and placement, as well as the influence of shear stress from hydrodynamics and aeration, define the process's possible effects on cells and the process. Determining the pressure inside the bioreactor is also an important parameter. The pressure is measured with a sensor connected to the bioreactor. A gas insertion lockout strategy is put in place if the pressure rises due to clamp-on or clogged vent filters as a result of excessive foaming, for example, due to mishandling of the bioreactor. As a result of excessive foaming due to misuse of the bioreactor, the ventilation filters are clogged, and therefore, when pressure increases, the A gas addition interlock strategy is applied. All these critical parameters are determined and controlled in bioreactor design. A signal from each probe is evaluated and accordingly the system is regulated at the desired level [8].

Shear stress has an effective effect on tissue function and viability. There are different values for the maximum sustained shear stress for each cell type. The high shear stress generated on the scaffold surface by a fluid flow can strip the attached cells, in which case tissue growth can be significantly slowed down compared to static cultures. Simply put, the fluid flow affects the shear stress, the orientation and function of the cells. For example, it has been observed that shear stress affects endothelial cell proliferation and directs them downstream (Fig. 9.9) [69, 123].

Although the operational process of sterilization varies little depending on the organism, it must be carefully adjusted to the bioreactors' geometrical design and material composition. With the determination of aseptic parameters, the sterilization procedure of the bioreactor is applied [135].

Tissue engineering bioreactors are mostly laboratory-scale (lab-scale) bioreactors that involve tissue production and tissue modeling. Experiments require multiple samples which are conducted in T-flasks or spinner flasks in incubators to observe cell growth and to perform substrate or product assays. Many tissue cultures are

performed in lab-scale as experiments require relatively low numbers of cells. In this scale, T-flasks with a surface area ranging from 25 to 225 cm^2 are used. The maximum number that flasks with a surface area of 225 cm^2 can reach is 1×10^7 per ml. The scale-up process is accomplished by gradually increasing the culture from the lab-scale to the industrial scale. This method gains functionality by adapting directly to the environment in which the cells are transferred and proliferating [21]. The scale-up is divided into two categories: scale-up in suspended cultures and scale-up in monolayer culture. In monolayer culture, cells proliferate by attaching to the flask surface. Therefore, it is necessary to increase the surface area and medium volume to scale-up monolayer cultures. Monolayer cells are more difficult to scale than suspended cells. Transfer of cells to the new medium cannot be accomplished as a simple fluid transfer, as the cells must be released from the substrate mostly using an enzyme. This consists of a highly variable and contamination-prone process that is labor-intensive in large-scale operations [51]. The advantages of monolayer cultures are ease of medium exchange, washing and cell perfusion, high production of pharmaceutically important components such as hormones, vaccines, insulin and interferon, and repeated use with different cells and mediums with the same experimental setup and equipment. The disadvantages are that it is tiring and costly, requires a lot of free space, cannot effectively monitor cell growth, and is difficult to measure important process parameters. The scale-up of monolayer cells can be performed in roller bottle culture, roux bottle culture, multi-surface culture, microcarrier culture, fixed-bed reactors, fluidized-bed reactors, and hollow-fiber reactors [125]. The scale-up is much simpler and more controllable for cells growing in suspension, as stirred vessels show similar design properties at all scales. Scaling-up the suspension culture is accomplished primarily by increasing the culture volume. Spinner flasks (100–1000 ml) and bench-top bioreactors (1–50 L) are used in lab-scale for the development of suspended cultures. After the reproducibility and repeatability of this bioprocess is possible and the process parameters are optimized, a pilot scale process (50–10,000 L) is designed to maintain optimum operating conditions. After lab-scale and pilot scale studies are successful, the plant scale (> 10,000 L) is designed for co mmercial and large-scale production (Fig. 9.9) [92]. By scale-up, it is aimed to successfully transfer the optimum conditions obtained in small-scale bioreactors to large-scale bioreactors. Scale-up studies are very critical and indispensable in order to create suitable parameters and conditions to change the scale without harming the kinetic behavior and growth performance of cells. However, the kinetic behavior of cells is significantly affected by local environmental conditions such as temperature, pH, DO, and nutrient concentration. Therefore, small-scale studies may tend to overestimate the process performance at larger scales if inconsistencies in scale-up are not resolved. For this purpose, environmental conditions and parameters should be kept under control and constantly monitored. This is done taking into account physical, biochemical and bioprocess factors. Physical factors include mixing parameters, heat and mass transfer, power consumption, DO, temperature, pH and shear stress. Biochemical factors are mainly media components and their physicochemical properties and concentrations in the

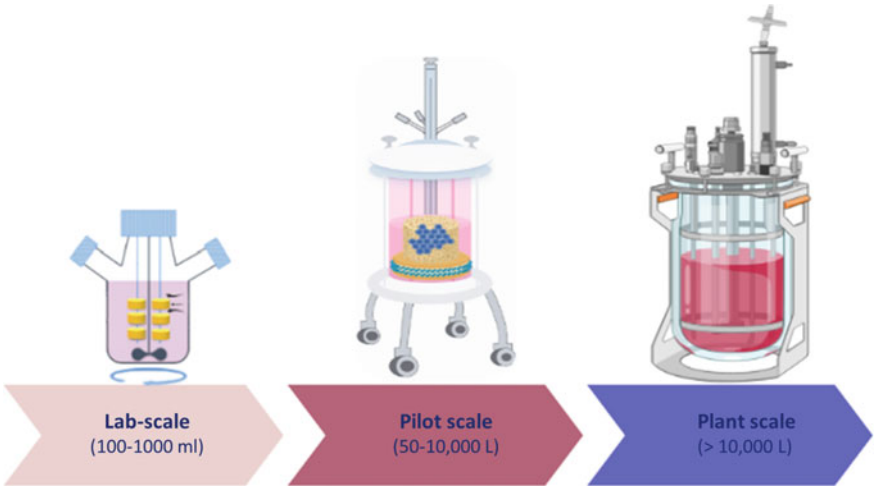

Fig. 9.9 Scale-up of bioreactors from lab-scale to plant scale. Created with Biorender (https://app.biorender.com/)

bioreactor. In addition, bioprocess factors such as pre-culture conditions, sterilization quality, and inoculation rate also determine how the scale will be administered successfully [138].

The conventional method used to scale up the bioreactor includes determining the geometry of the bioreactor, the stirrer speed, and the aeration rate of the large-scale bioreactor, taking into account the experimental results of the lab-scale bioreactor. The most widely used method of scale-up is to determine the dimensions of the large-scale reactor while maintaining the geometric similarity of the bioreactors. After the volume of the large-scale bioreactor has been determined, its geometric properties such as tank height, tank diameter, and agitator size are estimated using certain predetermined and accepted ratios and calculations. Typical bioreactors are cylindrical and designed to have a height-to-diameter ratio of 2/1–3/1. This ratio can be used as one of the simplest scale-up strategies. However, this ratio may not be so simple when applied to reality. Enlarging the bioreactor diameter by 5 times and keeping the height-to-diameter ratio constant will increase the reactor volume by 125 times, which undoubtedly makes the production of 3D textures on a larger scale quite different. Empirical correlations are needed to determine impeller speed and aeration rate and to keep the parameters related to the change in scale constant. Evaluation of the impeller speed is accomplished by keeping agitation power input per unit volume, volumetric oxygen mass kLa, or impeller tip speed constant. The aeration rate can also be determined by parameters such as equal superficial gas velocity, specific gas flow rate or gas flow number. In scale-up, one or more process parameters are kept constant by the engineers, estimations on other parameters are made accordingly and strategies are created in this direction [51, 92].

In summary, scale-up techniques in bioreactors bring some problems that need to be overcome. These problems are parameters that need to be optimized such as operating time, reactor capacity, oxygen, pH, temperature, gas exchange, mass transfer, continuous monitoring, product recovery, control of secondary processes, depletion of nutrients and oxygen, formation of toxic metabolites and production efficiency. The potential applications of bioreactors in tissue engineering can be better achieved when working with reproducible and repeatable systems with high degree by optimizing scale-up parameters and conditions.

Application of Bioreactors

Tissue engineering of all 3D tissues requires homogeneous cell distribution to develop homogeneous tissue [108]. With bioreactors, the biomechanical and biochemical environment that is effective in cell and tissue growth can be provided in a controlled manner. Therefore, functional cells and tissues can be grown suitable for transplantation using bioreactor technology (Fig. 9.10). These systems' major goals are to maintain ideal gas and nutrient concentrations in the culture medium, ensure homogeneity of cell distributions on 3D scaffolds, and expose the developing tissue to the similar physical stimuli. In vitro bioreactor systems based on controlled management of cell culture parameters ensure high reliability and reproducibility of experiments. Additionally, unlike bioreactors, static cultures on plates or flasks do not offer a flexible environment for studying cell-scaffold interactions under various pressure settings [139].

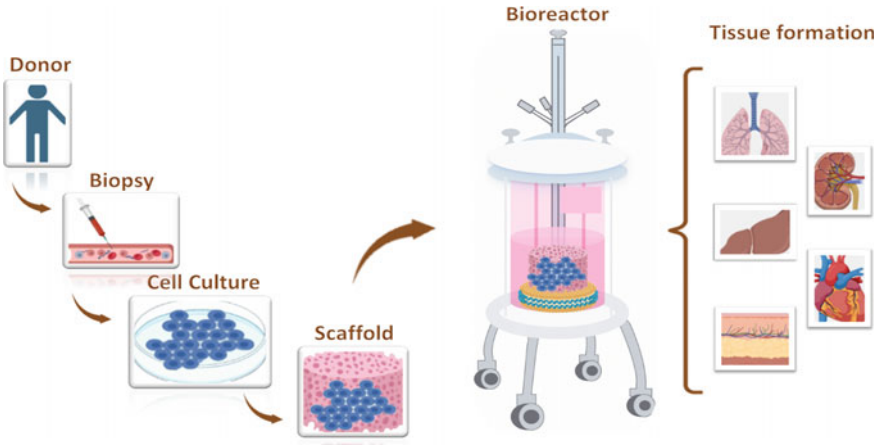

Fig. 9.10 General scheme of a tissue formation through a bioreactor. Created with Biorender. (https://app.biorender.com/)

To overcome the difficulties with large-scale cell production, big and uniform cell development in bulky tissue, high-efficient nutrient supply, important environmental stimulation delivered to cells, as well as metabolite removal, tissue engineering uses bioreactors [179]. The purpose of using a bioreactor can be listed as to promote cell proliferation, development, and placement within the scaffold to promote maturation and in vitro simulation of physiological or pathophysiological dynamic conditions [94].

Cell biology and tissue engineering studies require multiple isolations to maintain the cell source, cell expansion, cell viability, and long-term phenotypic stability. The standardization and quality control of biomanufacturing in this context is the next step that can make bioengineering a regular application in clinics [29].

As a result, bioreactor culture is required in several fields of tissue engineering. Bioreactors have been used in many different applications including whole organ, bone, skin tissue engineering [97]. In this section, use of bioreactors in tissue engineering of the gastrointestinal (GI) system, musculoskeletal, neural, cardiovascular, bladder, uterine, cornea, kidney, lung tissue will be examined.

Bioreactors for Gastrointestinal System Tissue Engineering

The GI tract is a complex system that involves the integration of different cell elements, immune, absorption, secretory, and motility signals. Intestinal motility, which is the process of coordinated contraction and relaxation of the smooth muscle in the GI tract, is a crucial aspect of intestinal physiology. This process, also known as peristalsis, occurs in various patterns of contraction and relaxation. Disruptions in peristalsis cause various GI diseases and disorders. Cell–cell interactions and GI disorders were understandable using 3D bioengineered models [29]. The cultivation of intestinal cells and tissues in dynamic bioreactor systems using 3D techniques is used to develop alternative treatments for intestinal diseases and to represent intestinal microenvironments in vivo. In a 2018 study by Zhou et al., a multifunctional bioreactor system containing pre-epithelized 3D silk scaffolds in a dynamic culture medium was designed for in vitro engineering of human intestinal tissues [184]. One notable example of bioreactor application is the development of gut-on-chip, which can simulate some human physiological features in a precise and controlled way [66, 95, 173].

Considerable attempts have been made to replicate the dynamic microenvironment of the gut. The perfusion bioreactor enabled to cultivate intestinal organoid units on biodegradable tubular polymer scaffolds that are compatible with live cell attachment. This method has been successful in maintaining organoids for up to two days, with potential implications for the long-term cultivation and bioengineering of intestinal cells [75]. Another bioreactor model, an electro-reactive elastomeric membrane utilized for in vitro modeling, imitate the mechanical patterns of intestinal tissue's contraction and relaxation cycles [22]. Pulsatile perfusion bioreactors have been employed to enhance the production of smooth muscle cells (SMCs)

and collagen in 3D PCL scaffolds which were exposed to pulsatile stretching and shear stress for up to eight weeks [70]. Bioreactors have also been employed to induce differentiation of adipose-derived stem cells into SMC of decellularized scaffolds that increase SMC phenotype expression and examine their contractile phenotype on collagen gel coating.

Bioreactors for Musculoskeletal Tissue Engineering

Musculoskeletal tissue, comprising cartilage, bone, skeletal muscle, ligament and tendons, may experience dysfunction as a result of various factors such as trauma and natural injury. Tissue engineering ensures a practical solution to the limited availability of natural implants and the inadequacy of current treatment methods for musculoskeletal tissue defects. Engineered constructs must be kept under sterile conditions and suitable stimuli that mimic the natural tissue with biochemical and biomechanical settings. Several types and configurations of bioreactors have been developed for the renewal of musculoskeletal tissue, such as spinner flasks, RWV, flow perfusion systems, and mechanical loading devices. Bioreactor designs that utilize dynamic flow (for cartilage and bone tissue) and mechanical cyclic stretching (for tendons, ligaments, and bone) are among the most commonly preferred for orthopedic tissue. There are several bioreactors available for culturing musculoskeletal tissue [1]. In addition to providing an in vitro environment that simulates in vivo conditions for the tissue growth bioreactors are important in tissue engineering also by enabling systematic investigations of the living tissues responses to a wide variety of mechanical and biochemical signals [5].

Numerous bioreactors are designed to apply mechanical stimulation along a specific direction, which enables the growth of oriented 3D muscle bundles capable of contraction. This approach results in the production of in vitro constructs with aligned muscle fibers that mimic the anatomical structure of skeletal muscles. However, the development of bioreactors that provide biaxial or radial stimulation is less common in the literature. While these types of bioreactors are less prevalent, they could offer unique advantages for the engineering of complex tissues and organs that require multidirectional mechanical stimulation. Further research and development of such bioreactors may lead to new approaches for the engineering of tissues with intricate anatomical features and functions. In the study by [158] a bioreactor capable of mechanical stimulation of porcine derived diaphragmatic scaffolds in a radial manner was designed to promote alignment of cell and muscular fiber development in clinically relevant diaphragmatic constructs [158].

For example, the RWV bioreactor has been tested to improve transportation of nutrient and promote tissue growth and differentiation in cartilage tissue engineering applications. The RWV bioreactor ensures a suitable hydrodynamic environment for cartilage tissue growth and phenotype differentiation [133]. Flow perfusion bioreactors are a viable culturing technique for bone structures. Interstitial flow plays

an important role in bone homeostasis. Bone grafts designed for accomplished cell stimulation, nutrient transport, and bone regeneration must be adequately perfused.

Bioreactors for Neural Tissue Engineering

Central nervous system (CNS) and peripheral nervous system (PNS) have limited self-renewal capacity in mammals, and disease and injury go about with persistent lack of functionality. Thus, CNS and the PNS renewal and renovation have significant challenges in tissue engineering [15]. The regeneration of damaged tissue is blocked because of the formation of astrocytes and scar of glia in the CNS. Mainly, research has focused on preventing further damage and stabilizing the affected area. But there are also studies focusing on repair processes to improve healing of loss functions related with CNS damage [142]. Recent neural tissue engineering strategies are developing for CNS and PNS tissue regeneration as potential treatments. Nervous tissue requires strict culture conditions and is even more difficult to induce differentiation and integration [152]. One of the most recent approaches often used to culture NSCs in vitro is the use of bioreactors in which biochemical or biological processes are tightly controlled and closely monitored [132]. In the study by Sun et al. [152], an approach using a closed-loop conduit bioreactor was used to introduce and culture Schwann cells on microfibers of longitudinally aligned viscose rayon and polystyrene model materials [152].

Bioreactors for Cardiovascular Tissue Engineering

Cardiac tissue engineering holds great promising approach for heart regeneration and modeling the pathophysiology of the human heart. Bioreactors are an essential tool in vascular tissue engineering and regulate physical and chemical parameters [114]. Bioreactors have been utilized for the amplification and differentiation of progenitor cells into the cardiomyocyte lineage [100]. Significant efforts have been made to develop functional and biomimetic cardiac structures. A number of bioengineered heart valve structures have shown encouraging results reaching clinical trials. In addition, small myocardial grafts have been well engineered using 3D bioreactors that provided precise control of specific stimulation parameters [55, 109].

Recently, a number of bioreactor systems have been developed in cardiovascular tissue engineering that mimic mechanical and chemical stimuli in vitro. The designs of these bioreactors are primarily concerned with tissue engineering of heart valves and blood vessels.

Different types of bioreactors have been used to develop supereminence heart valve tissue constructs. For example, dynamic and hydrodynamic, rotating, pulsatile, perfusion, and controlled cyclic stretching are the frequently used bioreactors [4]. A conventional vascular bioreactor typically consists of four main components: a

cultivation room, an electric pump, a media reservoir, and a temperature controller. The equipment has been developed in vitro to mimic maintenance of blood flow balance. In 2018, Wolf et al. developed a compact, portable and versatile bioreactor system that enables cost-effective large-scale and centralized production of autologous tissue-engineered vascular grafts and then transport of implants to patients [169].

Bioreactors for Cornea Tissue Engineering

Corneal diseases and injuries are prevalent worldwide and can lead to vision impairment or blindness if left untreated. One of the treatment methods is to replace the damaged corneal tissue with a healthy cornea from a donor, but there is a limited resource for donor tissue [91]. There are several studies to develop a tissue engineered cornea, which could potentially decrease the need for donor tissue and result in fewer post-transplant rejection rates. Although it is not widely utilized for the cornea, bioreactors have been reported in studies to assist to repopulate decellularized corneas with cells or as a culture method after initial seeding [46]. Also, in a study, the use of different materials in corneal tissue engineering bioreactors was investigated, considering that culture configuration, autoclaving and material surface preparation are important factors affecting cell viability [113]. The use of a rotary cell culture system for repopulation has been demonstrated in the literature, thereby encouraging cells to colonize the scaffold as they cannot attach elsewhere [30]. In an another study, a more sophisticated bioreactor system, a dynamic culture system for epithelial repopulation that mimics the in vivo air–liquid interface, has been reported [171].

It is known that the protection of ex vivocorneas in an environment that mimics natural physiological conditions allows the measurement of corneal thickness and its connection with cell functionality [129]. Application of mechanical stress to the cells is a potential method to control the in vitro phenotype of cells. Research has demonstrated that placing cells in an environment that simulates in vivo stress conditions can lead to the development of functional tissue equivalents [97]. A bioreactor has developed to obtain the possibility of using ex vivo corneas for functionality testing [54]. This study aimed to evaluate the survival of cells and tissue preservation of tissue structure in porcine corneas stored in a bioreactor that regenerates an intraocular pressure equivalent transcorneal pressure gradient and regenerates the corneal environment [54].

The tissue engineering approach is not limited to the cornea but has also come a long way for ocular tissues such as the lens and retina. There is a clinical need for ocular tissue substitutes [72]. In studies involving the combination of retinal organoid production with bioreactor technology, a bioprocess using RWV bioreactors to culture pluripotent stem cells sourced retinal organoids has been reported [37].

Bioreactors for Tissue Engineered Uterine and Bladder

Uterine histoarchitecture is highly complex due to the wide range of cellular and ECM molecules that include stratification of all uterine layers [3]. The process of decellularized tissues and organs, which involves the complete elimination of cellular components by forming a scaffold while preserving the structural, mechanical and biological properties of the ECM, is one of the most important stages of tissue engineering. Removal of cellular components is important to prevent immunogenicity. Maintaining the vascular network is important for the nutrient and oxygen supply to the uterus. Recellularization of the ECM construct is the next step of tissue reconstruction. To recellularize the organ, endothelial cells are perfused by the vascular network of the scaffold, usually through a bioreactor system, and must remain viable to induce cell growth in a controlled manner [3]. Although in vitro tissue growth seems to be successful in different scaffolds, it cannot show the same mechanical effects when transferred to an in vivo environment. This can because contraction forces in vitro cannot mimic the compression forces exerted by the surrounding environment after implantation. Additional mechanical properties necessary to enhance the urological outcomes of transplantation of cell seeded scaffolds can be achieved with the use of an in vitro bioreactor [148].

Similarly, in the field of bladder tissue engineering, simulating the normal physiological functions of filling and excretion with an in vitrobioreactor can improve the additional mechanical performance, tissue organization and maturation required to improve functional outcomes after implantation [139]. In the study by Niall F. Davis and Anthony Callan, a bladder bioreactor which consists of sealed pressure chamber with a pressurized gas containers, transparent window and silicone tubing was designed to physiologically mimic bladder dynamics [32]. Another widely used bioreactor to mimic bladder physiological conditions such as pressure, the modified BOSE BioDynamic® bioreactor, has been used in different studies in the literature [26, 90, 155].

Bioreactors for Kidney Tissue Engineering

The global prevalence of chronic kidney disease is increasing, and its therapeutic options are limited to peritoneal-dialysis, hemodialysis and kidney transplantation. Kidney transplantation is the most appropriate treatment as it improves long-term survival and is cost-effective compared to long-term dialysis. However, an insufficient number of donors is a major obstacle. In order to overcome this obstacle, the concept of creating an optional functional kidney graft using patient-specific stem cells has emerged and progress has been made in the last 10 years [163]. Tissue engineering has emerged as potential solutions to address the challenges in restoring kidney function. The kidney is a highly intricate organ comprising more than 30 distinct cell types with each type meticulously organized and functionally separated to create numerous

nephrons, the fundamental working units of the kidney. Therefore, various types of 3D kidney structures have been developed using appropriate scaffolding systems and cell sources to replace such complex kidney tissues and restore kidney function [73].

Both organoid-based and decellularization-based construction strategies of stem cell types, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have been widely studied for kidney tissue engineering [183].

To develop functional bioengineered kidneys for transplantation, an efficient recellularization strategy must be established. A recent approach for kidney tissue engineering involves the use of a bioreactor system that combines a cell infusion and perfusion culture system. This system can utilize various pumps such as a syringe pump, peristaltic pump, or pulsatile pump to allow for continuous cell infusion. The bioreactor system can also promote cell viability, nutrition, proliferation, and differentiation within the scaffold. The study by Song et al. summarized the production and transplantation of a rat kidney structure into rats using decellularization/recellularization techniques with a perfusion bioreactor system [147]. Briefly, the study involved, a decellularized rat kidney recellularized using human umbilical vein endothelial cells via the renal artery and rat neonatal cells via the ureter. Negative pressure was applied over the entire kidney chamber, followed by arterial perfusion culture in a bioreactor. The researchers were able to achieve a high recellularization rate, with 70% glomeruli present in the bioengineered kidney. In another study by Peloso et al., the decellularized kidney was recellularized in a customized pulsatile perfusion bioreactor providing optimal cell culture conditions [119]. Przepiorski et al., on the other hand, developed a strategy using spinner flask bioreactors to produce kidney organoids from iPSCs that is simple, strong, cost-effective, and allows large-scale organoid production [124]. Spinner flask bioreactors have been shown to enhance nutrient and oxygen perfusion [98, 115].

Bioreactors for Lung Tissue Engineering

Lung tissue engineering is an area of interest that promises a potential option for transplantation and pulmonary research. Lung biofabrication relies on seeding cells into a cell-free organ scaffold which is then cultured in a specialized bioreactor. Cell-free lung scaffold is achieved through conventional procedures that utilize physical, enzymatic and chemical agents. Similar to other organ tissue engineering, lung progenitor cells, autologous bone marrow/adipose tissue-derived MSCs or iPSCs are used for the biofabrication of the lung. A specialized bioreactor is employed to create an environment for circulatory perfusion and mechanical ventilation with physiological parameters to support the growth and function of the lung [45].

Significant advances have been made in bioreactors for lung engineering, both at the micro and macro scale. These are closed systems with pressure-controlled perfusion and ventilation. Ex vivo lung perfusion systems are systems developed for the protection and regeneration of the lungs [116].

Small-scale bioreactors have been improved to more accurately predict the lung environment than static air–liquid interface cultures. These bioreactors, described as "lung-on-a-chip", are to work as pharmacokinetic models for studying behavior of lung cells in drug discovery and studying drug toxicities [80]. Horizontal and vertical bioreactors have been developed for the upper respiratory tract (trachea, bronchus) [104]. For whole organ tissue engineering, intact lung scaffolds have been used and bioreactors have been developed for decellularization.

Bioreactors for Skin Tissue Engineering

Tissue-engineered organotypic full-thickness skin grafts may overcome delayed wound healing problems as they offer immediate coverage of the lesion by replacing both the dermal and epidermal layers of the skin. Many tissue-engineered skin graft studies have been successful, but since it has limited commercial utility in the clinic. The major limitation of available tissue-engineered skin substitutes is known as vascularization [58]. The application of bioreactors for more efficient graft production has been suggested. In a study conducted by Helmedag et al [60], the effect of a constant-flow bioreactor system on organotypic skin grafts was investigated and its use in the production of prevascularized organotypic full-thickness skin grafts was evaluated [60]. Also, there is a need for the development of bioreactors for tissue-engineered leather to advance its production to the clinic and for its production. Bioreactors must be able to culture skin structures at the air–liquid interface, due to the maturation of the epithelial layer to produce proper barrier function [85]. For the culture of metabolically challenging tissues, continuous perfusion with the medium is known to better support metabolic activity, rather than replacing new medium once per several passing days. In addition, the risk of contamination increases in long-term culture of tissue engineered constructs that require repetitive processing. Therefore, a closed system bioreactor at an air–liquid interface was designed for the production of autologous re-established skin, which would be suitable for both clinical and experimental use [151].

Conclusion

It is necessary to understand how complex physiological pathways work in the physical context of cells, tissues, and the interaction between different culture parameters to successfully continue tissue culture and tissue engineering applications and producing in vitro 3D tissues starting from isolated cells [79, 97]. Bioreactors have been used to produce vaccines and other drugs since the 1980s and have been evaluated to use in tissue engineering, allowing the application of robust, reproducible, and controllable culture conditions and making significant improvements in the design of the reactors [138]. Bioreactors stimulate cells to grow on a scaffold and produce an

ECM by mimicking their natural niche in vitro [121, 138]. The existence of complex biochemical, metabolic and mechanical stimuli and signals between cells and the cellular environment in tissue development requires an understanding of the specific cell behavior cultured at the molecular level to improve performance in tissue culture [121].

In tissue engineering, bioreactors focus on adequately mimicking the tissue's natural system, as described in the previous sections, so that natural tissue growth can be achieved. The reason why various bioreactor designs differ from each other specifically for the target tissue is due to the variety of tissues seen in the body [14]. In tissue engineering, especially micro and small-scale bioreactor designs allow examining and understanding the behavior of tissue cells at the molecular level, and to complete process development studies with microfluidic methods [97].

The main advantages of using bioreactors in tissue culture are improved mass carrying capacity, controlled and simultaneous traceable culture conditions, providing relevant stimuli in the environment, continuous media feeding and waste removal, reducing process steps to be processed, facilitating sampling and quality controls, preventing contamination and ensuring standardization [20, 164]. Thanks to these advantages, bioreactors are preferred, and progress is made in understanding the development of tissues in product development, research and clinical research. In addition to all these, the importance of bioreactors arises because the production under standard two-dimensional, static cell culture conditions cannot provide the stimuli and 3D space needed by the cells in the tissue regeneration process. The ability of bioreactors to simultaneously control and monitor many parameters (pH, O_2, CO_2, temperature) with sensors and detectors in tissue cultures provides significant convenience to researchers. However, all parameters should be optimized to meet tissue-specific physical, biochemical and mechanical requirements, and the right scaffold structures on which cells can expand should be selected. It is clear that no single design will fit for all tissues [138]. Identifying optimization requirements and equipment design for bioreactors requires an interdisciplinary approach [8, 121]. Considering the increasing technologies and application areas, bioreactors have an important place in the treatment of diseased or injured organs and tissues, in regenerative medicine, understanding tissues and cells, tissue engineering and accordingly, in the improvement and quality of human life.

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