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Recent Advances in Regenerative Tissue Fabrication: Tools, Materials, and Microenvironment in Hierarchical Aspects

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As part of regenerative medicine, artificial, hierarchical tissue engineering is a favorable approach to satisfy the needs of patients for new tissues and organs to replace those with defects caused by age, disease, or trauma or to correct congenital disabilities. However, the application of tissue engineering faces critical issues, such as the biocompatibility of the fabricated tissues and organs, the scaffolding, the complex biomechanical processes within cells, and the regulation of cell biology. Although fabrication strategies, including the traditional bioprinting, photolithography, and organ-on-a-chip methods, as well as combinations of fabrication processes, face many challenges, they are methods that can be used in hierarchical tissue engineering. The strategic approach to synthetic, hierarchical tissue engineering is to use a combination of several technologies incorporating material science, cell biology, additive manufacturing (AM), on-a-chip strategies, and biomechanics. Herein, in a review, the current materials and biofabrication strategies of various artificial hierarchical tissues are discussed based on the level of tissue complexity from nano to macrosize and the adaptive interactions between cells and the scaffolding surrounding the incorporated cells.

1. Introduction

The need for artificial organs and implants to repair and/or replace damaged organs and to correct congenital disabilities of patients is increasing.[1–3] If fabricated human-like organs or tissues are to be effective and to function optimally, they have to follow the hierarchical structure, which is a prerequisite to mimic the complex functions of the organs and tissues found in nature.[4] The hierarchical structures of tissue are the nature of living organisms, and the sizes of such structures range from nanometers to micrometers; furthermore, the definitive geometries of artificial organs and tissues give them many advantages, such as strength and biological interactions among subhierarchy levels.[5,6] Interactions between cells and of cells with the matrix surrounding them happen freely and on all sides in the hierarchical construct system.[7] Because the structures and functions of that construct system are essential, human-like artificial organs and tissues fabricated and used for regenerative medicine must mimic that hierarchical structure.

The final goal of tissue engineering is to create artificial tissue with the ability to perform biological functions. Nonetheless, materials are a critical part of successful tissue engineering for regenerative medicine. The materials' properties, such as size, stiffness, biocompatibility, and biodegradability, have to be considered during material selection for tissue engineering. Scaffolds have been described as an essential component underlying the successful formation of functional artificial tissues.[8,9] Therefore, with a proper scaffold material, an adequate microenvironment for cell proliferation, cell adhesion, and cell–cell interactions can be achieved.[10] Moreover, a suitable combination of scaffold materials and cells defines the functionality of the hierarchical tissue complex. The embodiment of cells during the fabrication process for cell-laden scaffolds and the incorporation of live cells into the fabricated scaffolds are integral parts of the organization of functionalized artificial tissues. For tissue regeneration, a scaffold, as the framework in tissue engineering, has to provide the features that can induce cellular and biophysical responses through the chemical compositions, elasticity, geometry, and ligand spacing of the biomaterials to modulate the behavior of cells.[11] The signaling cues from a synthetic polymer can be increased by protein incorporation into and addition of

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biomimetic factors from the extracellular matrix (ECM) to scaffolds fabricated using synthetic polymers.[12] The cellular physiology, including survival, migration, growth, and differentiation, is determined by the microenvironment provided by the cells' scaffold.[13] The biological interactions between the cells and the scaffold are combinations of receptor-mediated and mechanical-mediated signals, so-called mechanotransduction, that regulate the phenotype and the function of the cells. During this process, the cells will respond by converting mechanical stimuli into the ECM's biochemical signals to the cells' nuclei.[14,15] Mechanosensitive molecules in cells recognize those mechanical stimuli and initiate the mechanotransduction process.[16,17] Furthermore, biochemical molecules are synthesized in the cytoplasm after mechanosensitive molecules bind to receptors. Therefore, mechanotransduction, including mechanosensing and mechanosignaling, is the core of cell–ECM interactions that must be mimicked during tissue engineering for regenerative medicine.

For the engineering of tissues for the purposes of regenerative medicine, fabrication is an essential part of constructing a natural hierarchical structure, and many methods, for instance, microfluidics for organ-on-a-chip (OOC) and additive manufacturing (AM) strategies, including inkjet-based, extrusion-based, laser-assisted-based, and stereolithography-based 3D bioprinting, have been used. The microfluidics for OOC fabrication provides the minimal function of a tissue or organ and allows tissue and organ constructs to be explored under minimal conditions. This microfluidic approach to fabrication allows microenvironment tissue-based signaling to be simulated in a 3D tissue culture in a similar way as in in vivo applications. In contrast, 3D bioprinting yields improved cell organization with respect to classical tissue engineering with matrices throughout the scaffold and ultimately produces artificial tissues and organs with realistic, natural, hierarchical structures; moreover, 3D bioprinting allows those natural structures to be mimicked layer-by-layer, leading to the fabrication of different structures and different compositions of materials and cells in the different layers of the cell-laden scaffolds. However, the 3D bioprinting process requires a biocompatible ink that contains biological materials for the cells' scaffold and living cells for tissue fabrication.[18] If the complex, natural, hierarchical structures of tissues and organs are to be mimicked, the ultimate strategies, including material selection, the fabrication method, and the mimicking of their microenvironment, must be fully understood. Thus, a comprehensive discussion of this topic would include the materials to be used, the fabrication method, and the engineering of the fabricated scaffold to achieve the proper hierarchical structure so that the fabricated tissue or organ can be used for regenerative medicine.

2. Hierarchical Tissues

Living organisms are highly organized and structured, following a hierarchy that can be examined on a scale from single molecules to multicellular aggregates.[19] The definition of hierarchy encompasses objects with different dimensions and scales with anisotropic links between them. For instance, as the body's largest organ, skin comprises three layers, the epidermis, dermis, and the hypodermis, that vary significantly in their anatomies and functions.[20] The epidermis layer incorporates several layers, the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and the stratum basale or germinativum, as well as several residing cells, such as melanocytes, keratinocytes, Merkel's cells, and Langerhans' cells.[21] Tightly connected to the epidermal basement membrane, the dermis consists of papillary and reticular regions that comprise an interconnected network of collagen and elastin fibers produced by fibroblast cells.[22] Deep in the dermis, the hypodermis, as the deepest layer of skin, connects the skin to the bones and the muscles' underlying fascia.[23] Mimicking the hierarchical and unique structure of an organ is essential if an artificial organ with functions and characteristics similar to those of a real cell is to be constructed while considering the biological tissue requirements and the technical strategies (**Figure 1**).

The order of the highly structured, complex system of cells, the ECM, and cell signaling is the basis of the 3D microenvironment in natural tissues.[24–26] From the 1D biological building blocks to the 2D monolayer culture model of cells, the orientations of the cells in the ECM on the substrate's surface and of the cell-to-cell adhesions in the horizontal and the vertical planes lead to a complex 3D structure. In 3D, cells can initiate adhesions on all surfaces, but the surrounding matrix constrains cell migration.[7] In vivo, cultured 3D cells show more relevant behaviors and conditions, including cell adhesion, migration, mechanics, proliferation, differentiation, and responses to signaling molecules, than they do in the 2D cell model.[27–31] Due to cells' dimensionality, different native hierarchical levels need to be imitated artificially by controlling the functional molecular subunits that differ in size from nanometer to micron scale. In addition, time is considered an influential factor in the reactions of the properties of artificial tissues to alterations and interactions with the environment of the native tissue.[32,33]

In the hierarchical system of natural tissues, cells interact with similar and different cells to form communication between cells, and they play essential roles in tissue morphogenesis and function.[34–36] Cells interact with neighboring cells through direct and indirect mechanisms. Direct cell communication or cell–cell interactions occur through cell junctions and long-distance mechanical communication through the ECM.[37–39] Indirect cell–cell communication is conducted by soluble factors in the cell's microenvironment, such as basic nutrients and cell signaling molecules.[40] The ECM, which surrounds the cells in abundance, provides various cell adhesion ligands that will bind with the receptors on the cells' surfaces for transducing microenvironmental signals from or mediated by the ECM.[41,42] The ECM provides cells with biophysical properties and cues, including the structural mesh, the mechanical stiffness of the network, and variations in the macromolecules. The cells' behavior, performance, and functions in tissues are affected by the structural features of the ECM, such as its hierarchical order and the arrangement of the ECM fibers and pores that are formed in the occupying interstices of the ECM networks.[43–45] From tissue to tissue, the anisotropic structures of ECM fibers, which are hierarchically organized, can differ tremendously.[46]

Cell differentiation toward functional tissues, such as skin, bone, neural tissues, cardiomyocytes, etc., is modulated by the presence of cell signaling biomolecules (e.g., growth factors,

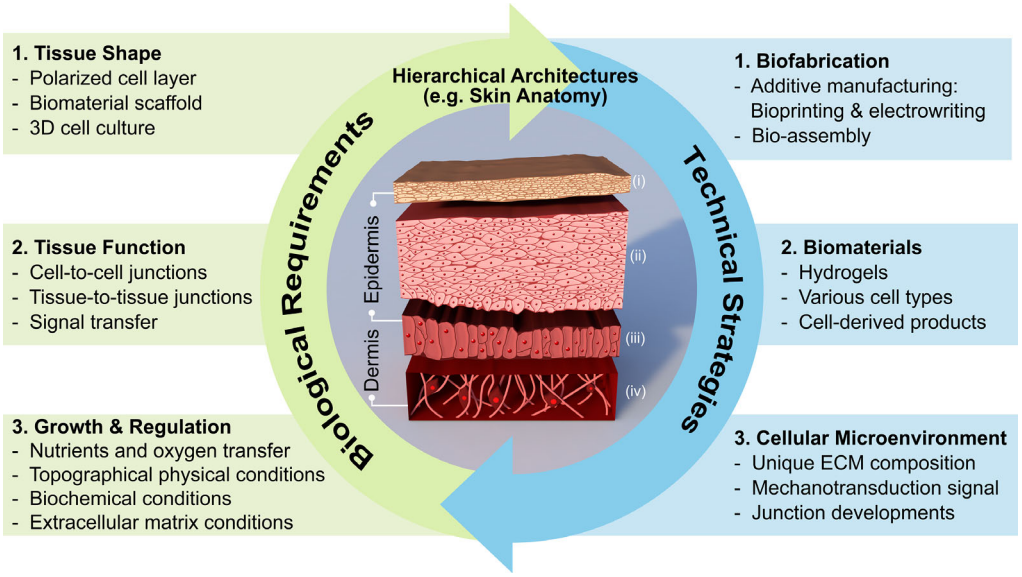

Figure 1. An illustration of hierarchical regenerative tissues. Skin is an example of a multilayered tissue with hierarchical architectures: i) *Stratum corneum*, ii) *stratum spinosum*, iii) *stratum germinativum*, and iv) ECM fibers. Technical strategies capable of satisfying biological requirements must be developed and utilized complementarily.

transforming growth factor beta, chemokines, cytokines, and genes) and by physical stimuli (e.g., physical forces, as well as cell–ECM and cell–cell physical contact).[47] In tissue engineering, if the hierarchy of biological tissue is to be fabricated, the signaling molecules, i.e., biomaterials with or without synthetic materials, must be incorporated through biofabrication to form a well-defined, biodegradable, porous, polymeric scaffold to induce desired cell responses from the seeded cells, leading to engineered tissues and organs.[48,49] Scaffolds play a vital role in this approach as they provide support for cell adhesion, and the cells proliferate, differentiate, and grow to form new tissues or organs. Ideally, scaffolds should possess a specific pore size that allows cells to spread, be biologically degradable and compatible, and have mechanical features.[50] Another way is to incorporate on a nano or microscale a single unit that can be utilized as a building block to construct tissue on a larger scale.[51,52] These modular units can be made using biofabrication methods, such as self-assembled aggregation, cell sheeting, microfabrication of cell-laden scaffolds, or 3D direct cell printing.[53–55]

The human body has the capability to regenerate its damaged tissue, but the degree of regeneration is limited due to several factors, including the type of tissue and its complexity, as well as the wound's size and severity.[56] An artificial tissue in regenerative medicine is a tissue made from a single biomaterial or from a combination of biomaterials and synthetic materials that systemically mimics the native tissue and is used for therapeutic applications to substitute for or restore the native tissue's function in the damaged area. The construction of artificial tissues with biomimetic forms and designs that aim to recapture the complex hierarchy and biofunctionality of human tissue is necessary if regenerative medicine is to be advanced and drug screening and disease models are to be developed.[57] An OOC using human cells is a promising development in regenerative medicine for recapturing the human physiological conditions and for integrating more than one tissue type to investigate a drug's efficacy and safety.[58] Furthermore, artificial tissues are tested in animal models as a preclinical test before being implanted in humans.[59] Therefore, materials with regenerative properties need to be made at different hierarchical levels. Because an organ-like microarchitecture must replicate the organ's structure, geometry, and functionality, both multiscale structural designs and functional building blocks are critical.

3. Fabrication Tools and Techniques

3.1. Effects of AM on Tissue Engineering

The fabrication technique plays an essential role in the success of hierarchical tissue engineering for regenerative medicine purposes. Several strategies, such as electrowriting, electrospinning, and AM, have been developed and being utilized to model hierarchical tissue engineering.[60–63] Electrowriting and electrospinning use an applied voltage to induce the collection of a stable fluid on a collector plate. The main difference between these two methods is the electrical instabilities caused by the applied voltage. In electrowriting, the function of the electrical instabilities is to generate a continuous fluid with a low flow rate on the collector plate. However, in the electrospinning method, electrical instabilities are used to drag a fiber onto the collector plate. Even though both methods involve the deposition of a fluid, neither is considered to be an AM process because of the dynamics of fiber deposition.[64] This dynamic process causes a fiber deposition instability in comparison with the AM process and differs from the AM process.

The AM process, or 3D printing technology, is a standard method for fabricating hierarchical tissues for regenerative medicine. The 3D printing method provides not only high precision

but also high cell organization in the scaffold. Moreover, in regenerative medicine technology, 3D printing is commonly used for scaffold construction to facilitate cell growth to replace and repair tissues or organs damaged by an accident or congenital disabilities. The 3D printing process not only provides the scaffold for cell proliferation but also constructs a cell-laden scaffold. Such a printing process that uses a biocompatible ink with a biological material and living cells is called 3D bioprinting.[18] The ink used in 3D bioprinting is a material consisting of living cells and a biomaterial or material with properties similar to those of the ECM components.[65] Layer-by-layer fabrication using 3D bioprinting can mimic a tissue's or organ's hierarchical structure, so an artificial tissue or organ can be designed by using a computerized 3D designer application to duplicate the complex structure of that tissue or organ hierarchically. Moreover, the integration between desirable and specific material properties has become a favorable approach for tissue engineering and regenerative medicine technology. Four basic approaches, which are discussed in the following sections , are commonly used in bioprinting fabrication: inkjet-based, extrusion-based, laser-assisted-based, and stereolithography-based bioprinting, which are shown in **Figure 2** and **Table 1**.

3.1.1. Inkjet Bioprinting

Inkjet bioprinting is a necessary bioprinting technology with the same basic principle as the conventional inkjet printing method, but with a biomaterial as the bioink, which is used in place of the conventional ink.[18,66] The printing device itself must also be modified to include a heater or piezoelectric actuator, which will allow the device to create a pulse that causes liquid from the cartridge to drop onto the substrate, thereby starting the fabrication of a specific scaffold based on the desired design.[18,67] Inkjet bioprinting, which is commonly used in tissue engineering and fabrication, is a fast printing process that is cost-effective, has high cell viability, and can produce several designs in a short time. However, this process has several problems: the size of the droplet is not uniform, the bioink can clog the nozzle, and temperature and pressure can place mechanical stress on the cells; these are summarized in Table 1.[18]

Artificial cartilage can be fabricated using inkjet-based bioprinting with a composite bioink made from polyethylene glycol dimethacrylate (PEGDMA) combined with gelatin methacryloyl (GelMA).[68] This composite bioink provides a suitable microenvironment for human mesenchymal stem cells (hMSCs) and allows the development of cell differentiation during the initial stage. Harnessing the crosslinking step to mimic the specific mechanical strength of natural bone and cartilage tissue can improve the mechanical properties of the 3D-bioprinted cartilage tissue's scaffold, increase the scaffold's strength through layer-by-layer photocrosslinking, and enhance the differentiation of osteogenesis and chondrogenesis in the hMSCs. The cells are well distributed throughout the scaffold when using inkjet bioprinting.[68,69]

Artificial blood vessels were successfully created using the inkjet bioprinting method. The speed of the printing process was found to play an essential role because a specific printing speed is required to create a fine line.[69] Collagen has also been used in bioinks for artificial skin fabrication using inkjet bioprinting. Autologous fibroblasts are used in the bioink because they perform better during therapy and cause neither infections nor complications. Such cells can reduce contraction and induce dermis epithelialization, leading to wound healing.[1] The in situ inkjet bioprinting process, which holds potential for fabricating the

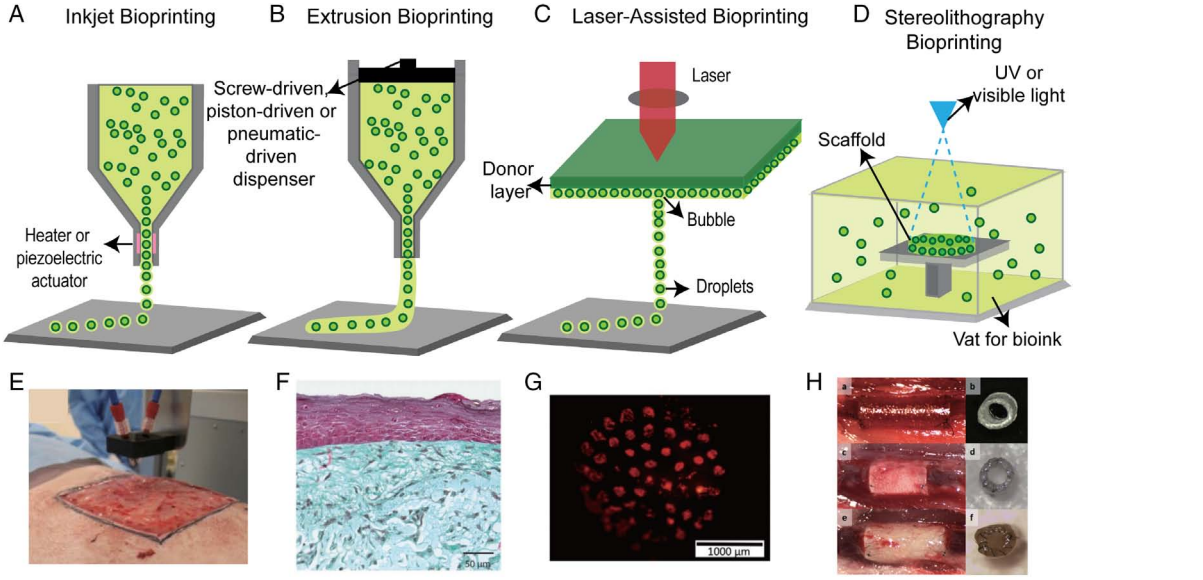

Figure 2. Different 3D bioprinting techniques: A) Inkjet-based, B) extrusion-based, C) laser-assisted, D) stereolithography-based, and E) in situ inkjet bioprinting processes. Reproduced under the terms and conditions of the Creative Commons Attribution license 4.0.[1] Copyright 2019, The Authors, published by Springer Nature. F) Morphological and histological features of artificial skin fabricated using extrusion-based 3D bioprinting. Reproduced with permission.[73] Copyright 2017, Wiley-VCH. G) Artificial bone disk fabricated using the in situ LAB process. Reproduced under the terms and conditions of the Creative Commons Attribution license 4.0.[84] Copyright 2017, The Authors, published by Springer Nature. H) Artificial vascular graft fabricated using stereolithography-based 3D bioprinting. Reproduced with permission.[87] Copyright 2016, Wiley-VCH.

Table 1. Bioprinting characteristics in hierarchical tissue-engineering applications.

No	Bioprinting approaches	Bioprinter characteristics	Application	Ref.
1	Inkjet bioprinting	Resolution: 5–500 μm High-speed printing process Inexpensive High cell viability Cells well distributed in the scaffold Issues in this bioprinting: Nozzle clogging Non-uniform droplet size Mechanical stresses on cells due to temperature and pressure	In situ inkjet bioprinting process of autologous skin cells on a wound's surface.[1]	[1,18,68,239,240]
2	Extrusion bioprinting	Resolution: 150–600 μm Different layers with different structures Use of different materials due to the use of several different printing heads High reproducibility Issues in this bioprinting: Low cell viability if high pressure is used Printing process not easy to control, combination between speed and pressure	Artificial skin fabricated using extrusion-based 3D bioprinting.[73]	[18,73,241]
3	Laser-assisted bioprinting	Resolution: 5–100 μm Nozzle-free printing avoids nozzle clogging. Noncontact process generates high cell viability. Fast with very high resolution Issues in this bioprinting: Possibility of toxicity due to the photoinitiator Limitations of printable materials	Artificial bone disk model.[84]	[81,84,242]
4	Stereolithography bioprinting	Resolution: 50–200 μm Nozzle-free bioprinting prevents clogging. High resolution High cell viability Issues in this bioprinting: Cell damage caused by UV exposure during printing process	Artificial vascular graft fabrication.	[66,87,243,244]

hierarchical structure of tissue due to its simplicity, easy control of the droplet size, and capability to produce specific designs, is shown in Figure 2A.

3.1.2. Extrusion-Based Bioprinting

Extrusion-based bioprinting, a fabrication strategy to construct a 3D structure layer-by-layer both in the vertical and the horizontal directions, is the most popular bioprinting technique used in tissue engineering in regenerative medicine.[66] Moreover, extrusion-based 3D bioprinting is well known to be a promising approach for fabricating hierarchical tissue. Due to its ability to use different bioink materials to fabricate tissue structures layer-by-layer, tissue constructs with different shapes and tissue scaffolds can be fabricated. However, a printable bioink with favorable properties, such as suitable viscosity and mechanical properties, must be used to fabricate 3D-bioprinted tissue with good physiomechanical and biological properties so that cells can adapt and survive. Low stiffness is also a must for cell growth, and the degree of crosslinking must be sufficient both to support cell proliferation during the fabrication of new tissue and to transport oxygen and nutrients to the cells.[70] However, when using extrusion-based bioprinting, several issues, such as low cell viability when high pressure is used, can occur; moreover, the interplay between speed and pressure is not easy to control. These characteristics are summarized in Table 1. That extrusion-based 3D bioprinting has been used to bioengineer several parts of the human body, e.g., bone,[71,72] skin,[73,74] cartilage,[75–77] and adipose[78] and neural tissues,[79,80] is noteworthy.

Artificial bone and bone scaffolds have been bioengineered through extrusion-based 3D bioprinting with several biomaterials, and synthetic polymers, such as polylactic acid–hydroxyapatite (PLA–HA)[72] and polycaprolactone (PCL),[71] are common polymers used as bioinks. Artificial bone was fabricated through indirect 3D bioprinting via a molding process,[72] with the mold being fabricated using extrusion-based 3D printing and the artificial bone being fabricated using the molding process. Perfusion is an additional step introduced to produce a highly, evenly porous scaffold to generate higher cell distribution, cell proliferation, and cell viability. PCL with dimethyl sulfoxide (DMSO) as a solvent has also been used as a bioink because DMSO can increase the porosity of PCL so as to increase cell adhesion, migration, and proliferation. Different compositions of PCL and DMSO have been used to produce 3D-bioprinted cell-laden scaffolds exhibiting different cell viabilities, mechanical properties, and cell adhesions.[71]

Alginate, gelatin,[73] and silk fibroin (SF)[74] have been used as bioinks for skin engineering via 3D bioprinting with an extrusion-based approach because this method, as compared with an in vivo system, can generate the hierarchical structure and the composition of artificial skin by creating a dermis system with a composition and organization similar to those of a mature tissue's cellular structure, as shown in Figure 2F[73] and Table 1. This 3D-bioprinted artificial skin was first bioengineered as scaffold-free skin to have the full thickness with human skin cells. With this technique, artificial skin can be fabricated quickly, and the cells can be evenly spread over the 3D-bioprinted artificial skin.[73] In addition to the aforementioned, gelatin–sulfonate–SF bioink was used to 3D bioprint artificial skin with the foreskin fibroblasts from a child incorporated.[74]

Norbornene-modified hyaluronic acid (NorHA),[77] GelMA and polyethylene glycol-methacrylate (PEG–MA),[75] and alginate and poly (ethylene glycol) diacrylate (PEGDA)[76] are generally used as bioinks during extrusion-based 3D bioprinting because they have adequate properties for fabricating artificial cartilage. Furthermore, in regenerative medicine, extrusion-based robot-assisted 3D bioprinting with bioactive materials was used for cartilage bone restoration via an in situ printing mechanism.[76] In addition, NorHA composite ink is a material that can potentially be used for the fabrication of artificial cartilage because HA is the natural material in cartilage. Moreover, in situ photocrosslinking with visible light is beneficial for the fabrication of a dense cartilage structure, and a uniform strength is achieved because the crosslinking takes place in every layer. This fabrication method can produce high cytocompatibility with a high and even distribution of cell viability in the 3D-bioprinted cartilage scaffold.[77] The GelMA and the PEGMA in 3D-bioprinted cartilage support the creation of hyaline-like cartilage, which allows the formation of fibrocartilage.[75] A combination of collagen and vascular endothelial growth factor (VEGF) was used to fabricate synthetic neural tissue via an extrusion-based 3D bioprinting technique.[79] The 3D-bioprinted VEGF with fibrin gel supported the growth factor in the scaffold from collagen. Furthermore, neural stem cells (NSCs) were incorporated into a polyurethane composite bioink for neural tissue engineering.[80] The biodegradability of polyurethane makes it a potentially good choice for neural tissue engineering. Because polyurethane can be synthesized in different ways, how it is synthesized can affect the proliferation of NSCs. In vivo application to animal wounds showed that when the 3D-bioprinted NSC-laden polyurethane scaffold was used, the recovery was better than it was without that scaffold.

3.1.3. Laser-Assisted Bioprinting

Laser-assisted bioprinting (LAB) is generally known to be a nozzle-free bioprinting method that uses a high-intensity laser for cell patterning[66] with high speed and resolution while avoiding nozzle clogging.[81] In this method, which has four main components, the laser, the target, a biological component, and the substrate on which the bioprinted materials are to be collected, the laser is used as the source of energy required to print the biomaterials on the substrate.[82] The components used in 3D LAB bioprinting are two glass slides, one with a layer of a laser absorbing substance and the other with a layer composed of the biomaterial. The laser light passes through the laser absorbing material and causes it to evaporate locally so that the pressure drives a small amount of biomaterial onto the glass slide. A specific biomaterial pattern is generated by repeatedly moving the glass slides, one and then the other, to create a 3D structure.[83] The characteristics of LAB and the issues encountered during the fabrication process are summarized in Table 1. The two most common issues faced during LAB bioprinting are the possibility of toxicity due to the photoinitiator and the limitations of printable materials.

Different cell types can be constructed in one 3D pattern when using this LAB method because cells can be deposited with microstructure resolution through computer control. This method was successfully used for tissue engineering in artificial skin fabrication,[83] and multilayer skin for patients with skin damage was fabricated by combining collagen, NIH3T3 fibroblasts, and HaCat keratinocytes. This bioprinted skin was applied in vivo to form new skin tissues. Not only skin but also bone has been fabricated using the LAB method. In artificial bone fabrication, a bioink, which is laden with mesenchymal stromal cells (MSCs), collagen, and nano-HA as biomaterials, is used, as shown in Figure 2G.[84] MSCs are used because these cells are multipotent progenitors that can differentiate between various cell types.

3.1.4. Stereolithography-Based Bioprinting

Another well-known type of nozzle-free bioprinting is stereolithography-based bioprinting, which utilizes a digital micromirror to control the light intensity at a specific printing area to polymerize a light-sensitive polymer material.[66] Stereolithography-based bioprinting uses an epoxy resin, a thermoplastic elastomer, a biomaterial-based hydrogel, and some synthetic polymers modified to increase biocompatibility and biodegradability.[85] Because this bioprinting method uses UV irradiation, damage to the cell is possible. The details of stereolithography-based bioprinting are summarized in Table 1.

Stereolithography is a bottom-up 3D fabrication method commonly used in dentistry, where stereolithography-based 3D bioprinting is generally used to form an artificial denture base. However, nowadays, even artificial teeth are being fabricated by using this method to photopolymerize a methacrylate-based biomaterial resin.[86] The bottom-up layer-by-layer deposition of the material is adequate for constructing the hierarchical structure of a tooth, and such artificial teeth show good fracture resistance because the layer-by-layer photopolymerization that occurs throughout the 3D bioprinting increases the binding strength of the methacrylate. The quality of 3D-bioprinted artificial teeth is comparable to that of teeth fabricated using traditional methods.

Artificial bone was fabricated using stereolithography-based bioprinting with a polypropylene fumarate resin.[85] However, this bioprinting method requires a postcuring step that may cause the printed product to shrink. In this method, the size and the shape affect the 3D-bioprinted scaffold because the hierarchical shape and structure are vital aspects in determining the mechanical characteristics of artificial bone. Polypropylene fumarate can also be used as an ink in vascular graft fabrication via

stereography-based 3D bioprinting, as shown in Figure 2H.[87] A 3D-bioprinted vascular graft was tested in vivo for several months, as shown in Table 1, and 6 months after implantation in mice, the artificial vascular graft showed suturability and adequacy.

3.2. Microfluidics for Tissue Engineering

Because bioprinting provides an excellent avenue for tissue constructs, researchers around the globe are expending much effort to investigate other methods to provide advanced approaches to tissue modeling for regenerative medicine. Microfluidics was developed to manipulate fluids on a microliter scale by using capillary action, pumping, and electrowetting. Microfluidics has been used for lab-on-a-chip (LOC) applications to deliver reagents and analytes for various assay detection protocols.[88] After the first attempts to use microfluidics to fabricate a cell culture platform, it began to be used to fabricate platforms for tissue engineering.[89] For tissue modeling toward regenerative medicine, microfluidics uses OOC, which can be used for 3D tissue cultures that can capture certain aspects of entire organs and organ systems.[90–92] However, microfluidic technology is not currently capable of building whole or hierarchical tissue; nevertheless, it is a promising tool for modeling native tissues or organs with minimal function and provides researchers with the freedom to develop drug and tissue constructs, a very important step forward in regenerative medicine in vitro.[92] Crucial features of microfluidics that are the keys to tissue construction toward regenerative medicine are the ability to be used for culturing cells in a 3D structure and to simulate microenvironments for cell development.[93] Although microfluidic technologies have advanced the study of tissue engineering, microfabrication through the use of microfluidics is a priority step that still needs to be addressed.

3.2.1. Microfluidics in Hierarchical Tissue Fabrication

Microfabrication using microfluidics was initiated by the electronics industry to find a way to fabricate microelectronic chips. The most common method and material used in 3D microfluidics to fabricate 3D cell cultures are photolithography and polydimethylsiloxane (PDMS), respectively. Photolithography is a very reliable method for precisely creating a microstructure; however, it involves many processes, such as making a master, cleaning, depositing a thin film photoresist, and etching, which must be performed in clean room facilities. The use of PDMS in microfluidics has various advantages, such as transparency, which allows the biomaterial inside a microfluidic channel to be optically observed, and gas permeability, which allows the oxygen tension inside a microfluidic channel to be precisely controlled.[94]

The functionality of the device that a study requires for specific 3D tissue modeling has to be taken into account when designing the microfluidics. Also, the microenvironment must be controlled, biophysical stimuli must be included, and the physiology of the tissue cell must be captured.[58,92] For example, for microfluidics to be used to fabricate a single-organ culture, such as a heart, liver, kidney, lung, gut, skin, or brain culture, the microfluidic system requires some important components for inducing biophysical stimuli, such as hydrodynamic, electrical, and mechanical stimuli, and for controlling the microenvironment of the tissue being studied. The design of a microfluidics system for fabricating a multiple-organ culture (blood–brain, heart–heart, gut–liver, and liver–kidney–lung–neural) varies because the designed system must allow connections and cross talk between the organs, as well as the integration of biophysical stimuli. As a result, the design of a microfluidic system for multiple-organ interaction studies can be categorized into three types: static, unidirectional single-pass, and recirculation.[92]

The design of a microfluidic system for fabricating a static tissue culture is simple because a microfluidic channel for the flow of a common medium is not needed, but a chamber for storing cultured tissue is. This multiorgan cell culture platform consists of shallow microwells that are used to culture cells for multiple organs, such as the liver–kidney–lung, and for blood vessels.[95–97] In contrast, another culture system, the unidirectional single-pass tissue culture system, is designed for the purpose of connecting one organ chamber to another via microfluidic channels. These microfluid channels enable cross talk between the organs through perfused microvessels. However, cross talk is only possible for organs located downstream, not organs located upstream.[98] The microfluidic recirculation tissue culture system is designed to have continuous circulation of a common medium through multiple organs, mimicking the flow of blood through blood vessels (**Figure 3**A).[99–102] Compared to the static and unidirectional single-pass tissue culture system, this system has a well-established external pumping system and microfluidic channels to control the hydrodynamics and the drug transport. In addition, the fluids effluent from the upper and the lower parts of an organ can be directly transported to another organ and collected for further analysis (Figure 3B–D).[99]

Although PDMS is a common material that has been used for the fabrication of microfluidic channels because of its optical transparency, durability, autoclave sterility, and biocompatibility, the pristine surface of PDMS has to be modified to make the surface suitable for culturing the cells because the surface of PDMS is hydrophobic whereas it should be hydrophilic for better cell attachment. The hydrophilic surface modification of PDMS can be done by using a physisorption, oxygen plasma/physisorption, layer-by-layer, or immobilization method to coat the PDMS with a functional material.[103] Another issue with the use of PDMS is its high permeability, which allows hormones and small molecules, such as drugs, to be absorbed. This issue can be addressed by coating PDMS with a low-permeability material, such as a lipid-based or poly(glycidyl methacrylate) (PGMA) material or a material containing silica nanoparticles.[104–106] In addition to PDMS, other materials, such as a tetrafluoroethylene-propylene (FEPM) and styrene–ethylenebutylene–styrene (SEBS) elastomer, have been used for microfluidic fabrication to resolve the drug absorption issue.[107,108]

3.2.2. Microenvironment Stimulation in Microfluidics

Conventionally, cells are cultured in cell-culture dishes. However, the microenvironment cues in that platform cannot be controlled properly to establish a specific microenvironment

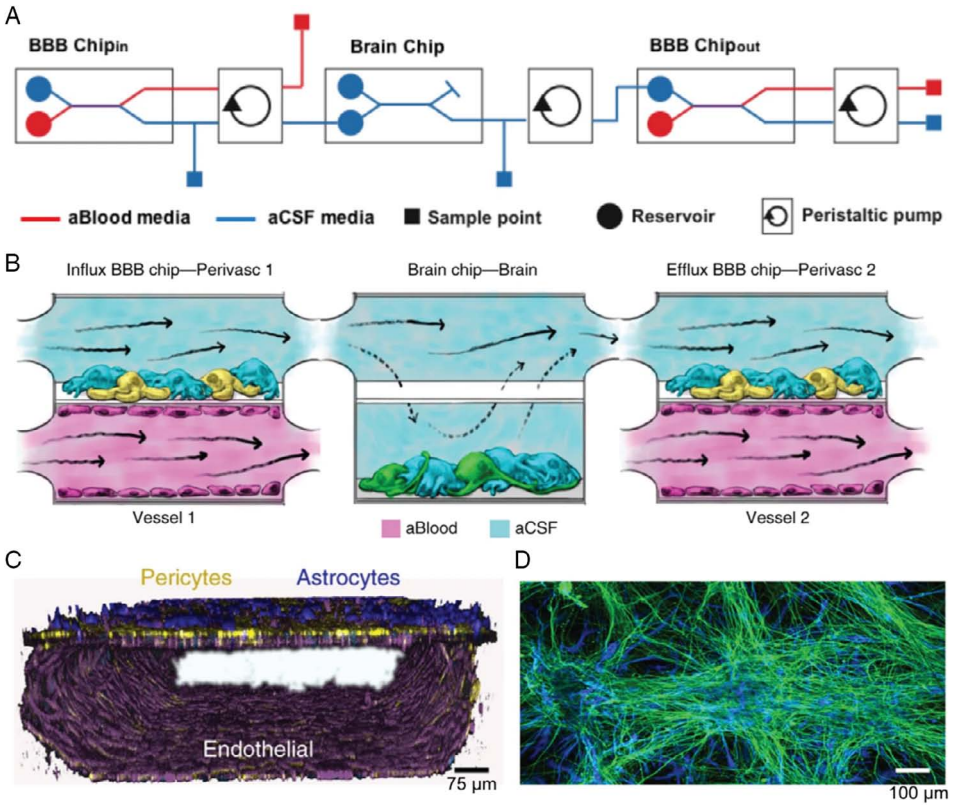

Figure 3. A,B) Schematics of a microfluidics system that allows continuous circulation of common media through the system and collection of the fluid effluent from organs, where aBlood refers to artificial blood and aCSF refers to artificial cerebral spinal fluid. C) The 3D pericytes, astrocytes, and endothelial cells are cultured in a microfluidics system. D) Confocal fluorescence micrograph of neurons (β-III-tubulin) and astrocytes (glial fibrillary astrocytic protein, GFAP) that are cultured together. The neurons and the astrocytes are green and blue, respectively. Reproduced with permission.[99] Copyright 2018, Springer Nature.

in which cells can grow and develop into mature cells. Moreover, this culture method fails to capture organ-level functions in vitro, which is crucial for facilitating drug development for regenerative medicine. In contrast, microfluidics can provide the features needed to mimic native tissue constructs and to control the microenvironment so that the physiological (hydrodynamic, mechanical, and electrical) and the chemical microenvironments needed to foster the function of native tissues can be simulated.[58,93,109–111]

Hydrodynamic stimuli, such as a fluid shear force and a fluid shear stress, can be generated in microfluidic systems by using external pumping at a specific flow rate.[112–114] The microfluidic channels act as vascular networks to produce solubility gradients, as well as autocrine and paracrine signals. The fluid shear force is very important for the correct biological functioning of endothelial cells because it activates surface receptors and associated signaling cascades.[115,116] Fluid shear stress, in contrast, is a periodic mechanical stress that can be enabled in microfluidic systems[117] and is a key differentiation parameter during the physiological process. The fluid shear stress was applied to mimic the environment of stem cells and to regulate their function and fate.[118,119] Jang et al. used fluid shear stress to control the microenvironment to investigate its influence on both the fluid reabsorption of aquaporin-2 and the orientation of actin cytoskeleton.[120] They induced this stimulus by using a syringe pump to cause the medium to flow through a PDMS-based microfluidic system that had been integrated with a microporous polyester membrane.

An electrical stimulus can be induced in a microfluidic system by using cooperating conductive electrode arrays in this platform.[121–126] Sun and Nunes engineered a system to stimulate an electrical microenvironment by integrating two platinum wires connected to two carbon rods.[127] Such a system allows tissue to mature in vitro. Another electrical microenvironment was constructed in a microfluidic system for continuously regulating the differentiation of neuroepithelial stem cells from patients.[128] The monitoring of the electrical microenvironment was achieved by using a programmable system to differentiate human neuroepithelial stem cells (hNESCs) into 3D networks of dopaminergic neurons on a microfluidic titer plate. Osaki et al. built a microfluidic system for the construction of 3D muscle bundles so that drug candidates can be tested and found the pathogenesis of amyotrophic lateral sclerosis (ALS) (**Figure 4**A–D).[129] They used electrical signals at different frequencies to stimulate muscle contractions to study the performance and the characteristics of motor neuron spheroids in a microfluidic system (Figure 4E).[129]

Chemical microenvironments are crucial for the development and function of cells and tissues. Microfluidics can be used for the precise delivery of a liquid volume so that chemical gradients

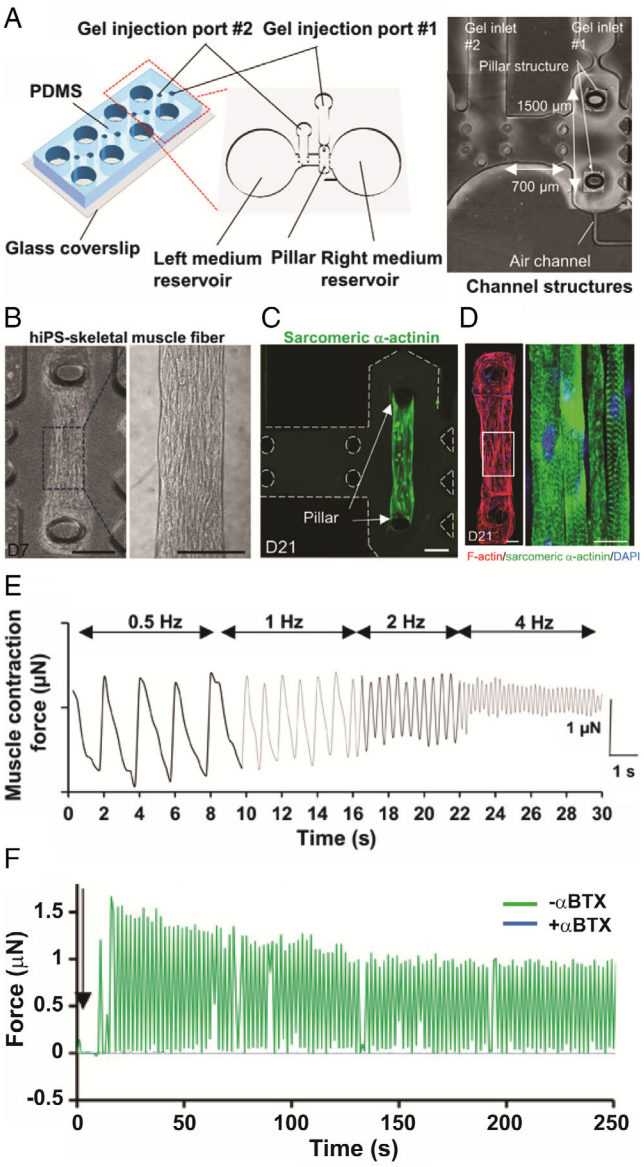

Figure 4. A) A microfluidic system for a 3D muscle cell culture in which the microfluidics surface is structured by using micropillars as a microenvironment to which cells can attach. The muscle bundle was grown in a microfluidics system, and the muscle fiber is attached the pillars on B) the 7th day and C) the 21st day. Scale bars = 200 μm. D) Confocal fluorescence micrograph of a muscle bundle showing the pattern of the cells and their sarcomeric structures. Scale bar = 50 μm. E,F) The variations with time of muscle contractions powered by various microenvironment stimuli such as electrical (at different frequencies) and chemical (glutamic acid at the 14th day) stimuli, respectively. Reproduced from.[129] Copyright 2018, The Authors, some rights reserved; exclusive licensee AAAS. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC).

can be maintained under the specific conditions required for tissue development.[130–133] For example, muscle contractions were produced by adding glutamic acid to a muscle cell culture that had been fabricated using microfluidics (Figure 4F).[129] In addition, the chemical concentrations in microfluidic systems can be controlled by reducing the permeability of the microfluidic material.[103,104,134,135] For instance, Gökaltun et al. reduced the permeability of PDMS by combining it with polyethylene glycol (PEG).[134] Also, their modified PDMS channel had a biocompatible, hydrophilic surface with long-term stability. The adhesive surfaces for the stem cell niche were prepared by patterning arrays of adhesive microwells. The adhesive surfaces were produced by using a programmable CO_2 laser to remove the coated polyvinyl alcohol.[136] Chuah et al. improved cell adhesion by coating polydopamine with collagen in a PDMS-based microfluidic channel to give long-term stability to a bone marrow stromal-cell culture.[137]

4. Materials for Hierarchical Tissue Fabrication

Materials have critical roles in the success of hierarchical tissue engineering. The variations of materials characteristics, such as size, biocompatibility, stiffness, and biodegradability, must be considered in material casting for tissue engineering purposes. A combination of and a correlation between scaffold materials and cells define the positioning of a complex, hierarchical tissue system. A scaffold made of suitable materials exhibits an adequate microenvironment for the proliferation of cells and generates cell adhesion and cell–cell interactions.[10] The various materials that are essential in hierarchical tissue engineering, such as synthetic materials, biomaterials, and cells, will now be discussed.

4.1. Biomaterials

The biocompatibility and biodegradability of biomaterials for a scaffold are critical aspects in tissue engineering. The chemical composition, the structure, and the properties of a biomaterial underlie the cell integration inside the scaffold and facilitate the scaffold's fabrication. Several kinds of biomaterials, for instance, natural polymers and decellularized extracellular matrices (dECMs), are used for tissue-engineering applications. Gelatin,[138–141] alginate,[138,141,142] chitosan,[143,144] SF,[145,146] and collagen[138,147] are common natural polymers with high biocompatibility and biodegradability that can be used for cell–scaffold purposes. The similar properties of gelatin and natural ECM provide the right conditions for cell migration and make gelatin an excellent natural polymer for the cell's scaffold.[138,141] The subtle behavior of gelatin in mimicking the ECM initiates cell proliferation, increases cell attachability, and generates high cell viability in the scaffold.[141] Moreover, gelatin is abundantly available in nature.[148] Based on the purpose, gelatin can be integrated with SF in the engineering of skin tissue with properties that resemble those of natural skin, as shown in **Figure 5**E.[74] Integration between the properties of gelatin and the specific fabrication processes will create a hierarchical network inside the gelatin scaffold and allow the fabrication of a porous structure to support cell proliferation.[149]

The gelatin process with alginate can be easily customized and modified so that other materials can be used. The lack of significant toxicity makes alginate a biomaterial with high biocompatibility for use in scaffold fabrication.[142] In mammals, due to enzyme deficiencies, alginate does not easily degrade, which can be overcome by using crosslinking modifications.[138] The use of a hybrid material, alginate modified using a dECM and methacrylate

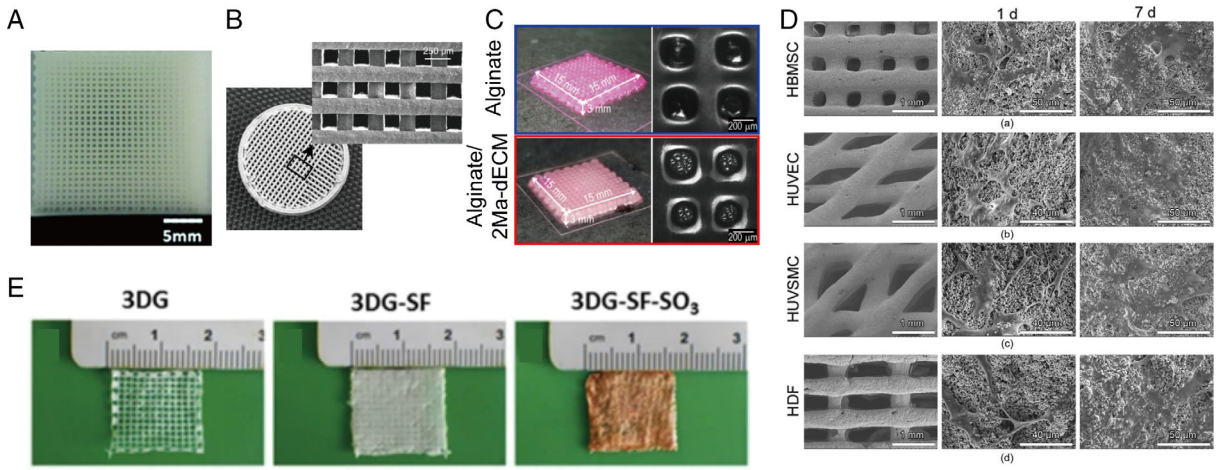

Figure 5. Biomaterials and synthetic materials for tissue engineering. A) The 3D scaffold from a PCL-based material for bone tissue engineering. Reproduced with permission.[71] Copyright 2020, RCS Publishing. B) The 3D-bioprinted micropores of HA for bone tissue engineering. Reproduced with permission.[151] Copyright 2007, Elsevier. C) The 3D-bioprinted alginate and alginate/2Ma-dECM-based bioink. Reproduced with permission.[237] Copyright 2020, Elsevier. D) Different cells cultured on different 3D-bioprinted β-TCP/HA scaffolds: cultures of human bone-marrow-derived mesenchymal stem cells (HBMSCs) (squares), HUVECs (parallelograms), human umbilical vein smooth muscle cells (HUVSMCs) (triangles), and human dermal fibroblasts (HDFs) (rectangles) after 1 and 7 days. Reproduced from.[238] Copyright 2020, The Authors, some rights reserved. Published by Elsevier LTD on behalf of Chinese Academy of Engineering and Higher Education Press Limited Company. Distributed under a Creative Commons Attribution NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND). E) The 3D-bioprinted gelatin scaffold (3DG), SF-coated 3D-bioprinted gelatin scaffold (3DG–SF), and SF-derivative-coated 3D-bioprinted gelatin scaffold (3DG–SF–SO_3). Reproduced under the terms and conditions of the Creative Commons Attribution license 4.0.[74] Copyright 2017, The Authors, published by Springer Nature.

reaction, leads to maximal mechanical properties, provides for cell proliferation, and makes fabrication easy (Figure 5C).

In the fabrication strategy for artificial bone tissue, natural materials, for instance HA and chitosan, are widely used to construct the hierarchical structure. HA exhibits a proper pore size for the growth of bone cells and has sufficient mechanical strength for bone engineering.[150] A suitable pore size can be achieved using sintering.[151] Figure 5B shows a HA scaffold achieved by sintering for bone tissue engineering applications. β-TCP-modified HA exhibits good biocompatibility with several cells in the cell culture and has sufficient porosity for tissue engineering, as shown in Figure 5D. In contrast, chitosan is a natural biomaterial that is easily extracted from the shells of shrimps and crustaceans. Its excellent characteristics, such as good mechanical strength, biocompatibility, and biodegradability, as well as nontoxicity, make chitosan a perfect candidate for medical and tissue-engineering applications.[143,152] In the fabrication of artificial skin, a natural polymer with excellent biocompatibility, environmental stability, and nontoxic response must be used. Thus, natural polymers with those characteristics, such as SF, are suitable for skin tissue engineering.[74] A scaffold made from a gelatin–SF hybrid material exhibits better wound healing due to the properties of SF, and the cells can be distributed evenly along the scaffold. Furthermore, SF is biodegradable and provides an adequate microenvironment for chondrocyte growth in cartilage tissue engineering applications.[145] The high rejection rate during the implantation process is the major drawback in SF-based tissue engineering.[146] Because collagen is often found in skin, connective tissue, cartilage, and bone as the ECM's main protein and provides an adequate microenvironment for cell proliferation,[138] it is widely used in tissue-engineering applications.[153]

4.2. Synthetic Materials

Because of the properties of natural polymers, with some modifications during synthesis, synthetic materials are excellent candidates for hierarchical tissue engineering. One of the common synthetic polymers with good biocompatibility and biodegradability is PLA. It is suitable in scaffold hierarchical tissue engineering of bone, neural, cardiovascular, and cutaneous tissues.[154–158] PLA is commonly used in artificial bone tissue engineering to fabricate a scaffold with a modified geometrical structure. The scaffold's pore size, which was between 0.2 and 0.35 mm, was sufficient for cell proliferation while still providing good mechanical properties for bone tissue engineering.[155] However, PLA-based materials have fewer sites for cell attachment and are, therefore, inadequate for cell adhesion.[154] Surface modification of PLA by using a plasma treatment can increase the attachment of cells to PLA.[158] Poly(lactic-co-glycolic acid) (PLGA) has adequate properties for dental treatment purposes and is used not only in dentistry but also for tissue grafts, sutures, bone scaffolds, and drug delivery systems.[159,160] Wang and colleagues enhanced the bioactivity of PLGA by coating it with a nano-HA material. They found the cell viability and proliferation to be higher than they were for PLGA without a nano-HA coating.[161] PEG can modify PEGDA, which is commonly used for body implants due to its biocompatibility and nontoxicity, for drug delivery, and for regenerative medicine purposes, by adding acrylate groups.[162,163] PEGDA has a low in vivo biodegradability, so a copolymer of PEGDA with poly(glycerol sebacate) acrylate (PGA) increases the degradation rate significantly compared with PEGDA without any modification.[164] PCL has an essential structure for bone tissue engineering and has good

biocompatibility and nontoxicity for medical implant purposes.[138,165] The good mechanical properties (high strength) and the large biodegradation rate of PCL allow its use for bone fabrication in tissue engineering (Figure 5A). If the PCL surface is modified using a plasma treatment and other polymers, such as collagen and poly(methacrylic acid) (PMAA), the attachment of cells on the PCL surface and the cell proliferation rate will be enhanced.[166,167]

4.3. Cells in Tissue-Engineering Applications

Cells are essential in hierarchical tissue-engineering applications, and cell–cell and cell–scaffold interactions are critical for tissue-engineering success. Suitable cells and microenvironment support for cell proliferation are a must. Various cell types have been used to fabricate skin, neurons, cardiac tissue, liver tissue, blood vessels, and musculoskeletal tissues.[168–171] The response of fibroblasts during ECM synthesis and their roles in controlling cell migration or settlement along the specific 3D structure of organs make them popular in tissue-engineering regenerative medicine applications. Incorporation of fibroblast cells with endothelial cells is a strategy for skin tissue regeneration, and autologous and allogeneic fibroblasts are frequently used for that purpose. However, compared to allogeneic fibroblasts, autologous fibroblasts are associated with better results, such as the low risk of rejection or cross infection and improved skin regeneration.[168] A hierarchical mechanism mediated by a transcription factor converts fibroblasts into induced neural cells.[171] Fibroblasts in cardiac tissue engineering maintain the ECM and provide a scaffold for cardiac cell proliferation and homeostasis.[168] Originally derived from liver tissue through the stem cell procedure, hepatocytes can potentially be used in liver regeneration therapy.[172] A 3D hepatocyte culture was able to maintain the liver's metabolic system as in an in vivo environment.[173] Human umbilical vein endothelial cells (HUVECs), which originally come from endothelial veins in the umbilical cord, are generally used for vasculature and angiogenesis studies.[174] HUVECs are generally used for in vitro studies because they are relatively easy to isolate.[175] Moreover, combining HUVECs with a suitable biomaterial is a promising tissue-engineering strategy for regenerative medicine purposes.

5. Biophysical Cell Responses in Tissue Engineering

The hierarchical scaffold in engineered tissue is important to provide the cells' microenvironment, including the ECM, biochemical soluble factors, a biophysical field, and surrounding cells, to give mechanical stimuli to the cells that elaborate many aspects of cellular physiology, such as migration, growth, differentiation, and survival.[11,13] The scaffold, regardless of whether it is a synthetic or a natural scaffold, modulates cell behaviors for functional tissue regeneration.[176] Mechanotransduction is a sensing and responding process of cells to mechanical stimuli that occur when cells attach to a biomaterial substrate's surface. The mechanical stimuli are converted into biochemical signals from ECM to the nucleus. This process involves a combination of receptor- and mechanical-mediated signals that will regulate the cell's phenotype and function.[14,15]

5.1. Mechanotransduction and Mechanosignaling

Mechanotransduction starts when integrin receptors in the cell membrane attach to the ECM components in the scaffold. Integrins, a superfamily of cell adhesion receptors, are heteromeric molecules assembled in α and β subunits that are noncovalently linked to various ECM components, and the combination of those subunits defines the ECM component and the cell type.[177,178] The extracellular domain of an integrin receptor binds to an ECM molecule while the intracellular domain interacts with cytoskeletal actin in the cytoplasm. Cellular binding to the extracellular domain of an integrin receptor is mediated by direct ligation to a specifically defined sequence of ECM molecules.

Integrins that bind to appropriate protein sequences tend to cluster on the cell membrane, which has a positive effect on linkage formation with the ECM and leads to concentrated configuration regions of integrins adjacent to the ECM. These links are known as focal adhesions (FAs), and they allow communication between the extracellular and the intracellular environments. The FA complex has two classes of mechanotransduction proteins that coincide as follows: proteins acting as mechanosensors by converting the conformation in response to mechanical stimuli and proteins acting as mechanoeffectors by recruiting downstream effectors in response to mechanical stimuli.[15,179] After integrins bind to ECM molecules, they bind to the cytoskeleton and promote intracellular reorganization, thus maintaining the cell's shape and internal architecture.[180] Integrin binding with the contractile actomyosin machinery is crucial to the substrate's mechanics and mechanosensing.[181] On the basis of molecular interactions, the tripeptide, arginine-glycine-aspartate (RGD)-binding integrin clusters in which β3 integrin binds to a number of components in the ECM and α2β1 integrin binds to GFOGER, an amino sequence mostly containing collagen molecules, were very extensively studied, and in tissue engineering, incorporating those integrin clusters into the scaffold was found to be a strategy with potential for enhancing cell adhesion.[182–184]

In the cytoplasm, β-integrins bind to the globular head domain of talin, a mechanosensor that activates talin's mechanoresponsive properties by introducing cryptic vinculin binding sites and mediating the formation of an F-actin and vinculin complex.[185,186] This activation process stimulates the association of cytoskeletal proteins, including vinculin, talin, alpha actinin, the Src family of kinases, and FA kinase (FAK), to form a FA complex.[187] Autophosphorylation activates FAK, which exerts its signaling functions via phosphorylation of downstream targets, such as the Src family of kinases and paxillin, resulting in the regulation of mechanotransduction and the dynamics of FA turnover.[187,188] The dynamic turnover of talin and vinculin is regulated by their activation states, thus activating downstream signaling to Rho-family GTPases. The activation of Rho leads to higher contractility and FA growth whereas the Rac activity increases the actin polymerization at the leading edge.[181] Cell adhesion through vinculin activates nuclear localization of a YAP (yes-associated protein 1) and its coactivator PDZ-binding motif (TAZ), which is regulated by the substrate's stiffness and the cell's geometry. Once this complex is activated and translocated to the nucleus, it modulates numerous gene

transcriptions.[189] The YAP/TAZ complex has been extensively studied as a critical regulator of stem-cell differentiation and cell proliferation, as well as organ overgrowth and survival, by pairing it with DNA-binding factors of the TEA domain (TEAD) family.[190–192] In tissue engineering, the effect of the scaffold on cellular behavior is mostly concerned with YAP/TAZ complex regulation in the cells.

Even though integrin is responsible for the adhesion of cells to the surrounding ECM, growth factors have been found to be involved in these integrin-dependent processes.[192] The sum of cell responses arising from growth factors depends not only on the type of growth receptor but also on the cell, the receptor type, and the intracellular receptor signaling pathway. The same growth factor molecule binding to the same receptor may induce different cellular responses in different cell types.[191] Cellular responses triggered by growth factor signaling include cell survival, migration, proliferation, and differentiation into a specific cell lineage.[191,192] A certain type of growth factor molecule represents a heparin-binding domain, which exhibits ECM binding through integrin to elicit a specific cellular response. Interestingly, the optimal stimulation of a growth factor mostly depends on integrin-mediated cell adhesion to the appropriate ECM. Many studies have revealed how to modulate the integrins and growth factor receptors in a bidirectional way, for instance, β1 integrin and epithelial growth factor receptor interactions in epithelial cells.[193] Cross talk between integrins and growth factor receptors (**Figure 6**) is crucial for sustaining and determining normal development and pathological processes in vascular biology. Growth factors, such as epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor, fibroblast bone morphogenetic protein-2, and VEGF, have been extensively developed by incorporating them with biomaterials for tissue engineering.[13,190]

5.2. Chemical Compositions and Manipulating Cell Responses

The chemical properties of the scaffold affect the behavior of stem cells to determine the cell's fate.[194–196] A collagen-I scaffold was found to induce mouse embryonic stem cells to differentiate into cardiomyocytes with or without a RGD peptide and in combination with laminin.[194,197] In contrast, when that scaffold was combined with fibronectin or laminin, embryonic stem cells were strongly driven to endothelial cell differentiation and vascularization.[194] On a synthetic polymer scaffold, such as a 3-butyrate-cohydroxyhexanoate (PHBHHx) or an alginate scaffold, the RGD peptide is conjugated to enhance cell attachment and to nurture chondrogenic differentiation of bone marrow-derived MSCs.[198,199] The combination of a natural ECM and a synthetic scaffold, such as a collagen nanofibrous and PLGA scaffold with recombinant fibronectin and cadherin 11 incorporated, was found to enhance the proliferation of MSCs, induce osteogenic gene expression (alkaline phosphatase, RUNX2, and osteocalcin) and hold potential for bone tissue engineering.[37] If desirable cell behaviors are to be achieved, control of the chemical composition of the scaffold is a crucial factor for providing biophysical cues to cells during functional hierarchical tissue assembly.

Every tissue has its own stiffness as defined by Young's modulus or its own elasticity; thus, in tissue engineering, matrix

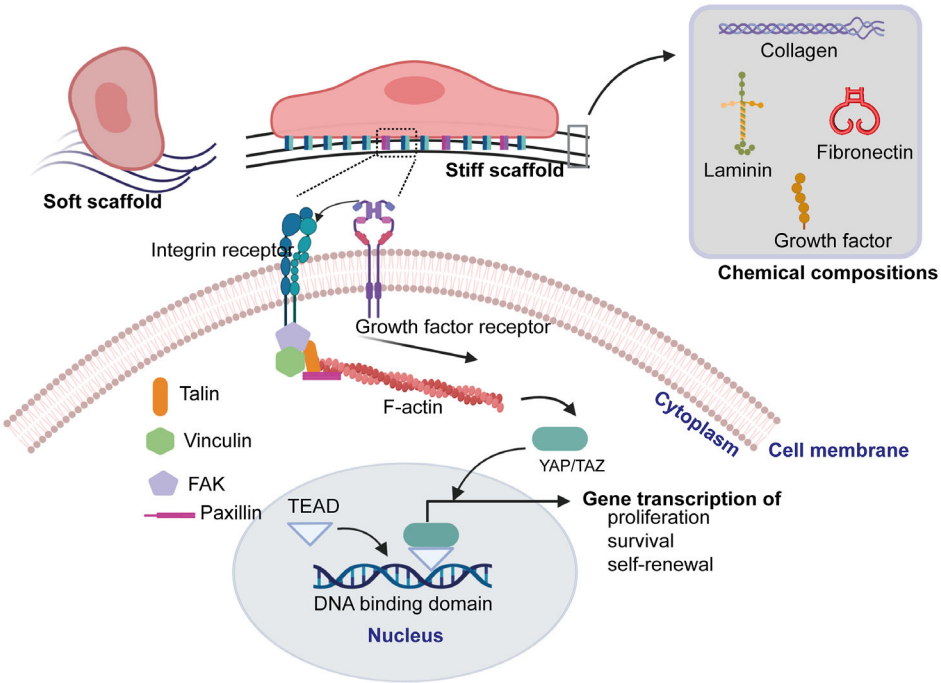

Figure 6. Schematic representation of the mechanotransduction signaling pathway involved in cell–scaffold interactions. This process starts at the FA site when integrin engages the scaffold with varying stiffness and chemical compositions (e.g., collagen, laminin, fibronectin, and growth factor) to assemble other proteins, such as talin, vinculin, FAK, and paxillin. This complex directly regulates F-actin assembly and dynamics and further activates YAP/TAZ complexes. These complexes then translocate into the nucleus to stimulate downstream gene transcription with TEAD coactivator transcription. This signaling propagates when the growth factor receptor directly links to integrin.

stiffness is important for determining cell responses to mimic the real conditions of tissue. The effects of substrate stiffness on cell migration and FAs were extensively studied to reveal the mechanical properties of the cell's response.[13,200–203] Cells can migrate easier on a soft or less-stiff substrate than on a stiff substrate.[203] Cell–scaffold binding generates a huge force from the cytoskeleton via an actin–myosin complex and guides mature FA and the high organization of the cytoskeleton on a stiff substrate (Figure 6). In contrast, on a soft substrate, cells cannot generate enough counterbalance to develop stress fibers.[204] Matrix stiffness influences cell morphology due to cytoskeleton organization: on a soft scaffold, cells are round while on a stiffer scaffold, cells tend to spread. The cellular responses to stiffness are clearly observed through nuclear translocation of the YAP/TAZ complex. The higher the ECM stiffness is, the larger the YAP/TAZ translocation to the nucleus via active transcription is.[189] In a stiff 2D hydrogel, the YAP/TAZ nuclear/cytoplasmic ratio is larger whereas it is smaller for a stiff 3D gel.[205]

Geometry and ligand spacing control play essential roles in orchestrating mechanotransduction, mimicking the tissue's length scale and directing stem cell differentiation.[41] Cell geometry controls the nuclear translocation of the YAP/TAZ complex to activate the transcription of some genes, and in the intermediate adhesion area, it can also direct the differentiation of MSCs.[189] Decreasing the size of MSCs by modulating the surface topography via nanopatterning was found to induce differentiation into an adipogenic lineage by increasing the FA and the YAP/TAZ nuclear translocation.[2] Cells with rectangle and star shapes preferred cell osteogenesis. In contrast, cells with square and flower shapes favored adipogenesis.[206] If the tissue's physiological length scale is to be mimicked, the spacing between adhesive molecules in the ECM micropattern needs to be precisely controlled.[41] A larger micropatterning space (>73 nm) reduces cell spreading and attachment, significantly limiting the configuration of FAs and actin stress fibers.[207] Moreover, another study showed that the local order of ligand arrangement influenced the integrin clustering and the cell adhesion induced by RGD ligands. When the substrate spacing arrangement is wider than 70 nm, cell adhesion is inactivated by the RGD nanopattern order while it is activated by the RGD nanopattern disorder.[208]

Taken all together, the cellular response to the microenvironment via mechanosignaling has been found to induce integrin binding to the scaffold. In tissue engineering, this biophysical response is increased by selecting a biomaterial with an appropriate chemical composition for scaffold fabrication, by modifying incorporated biological constituents, and/or by using the mechanical properties of the scaffold, such as its geometry, stiffness, and topography. Cutting-edge work on scaffold development addresses the fabrication of a controllable multicomponent structure that mimics a biological cell-derived scaffold[209] to support cell growth and control differentiation. Therefore, controlling all those factors to achieve the desired goal is crucial in tissue engineering.

5.3. Micro and Macroenvironments for Cellular Responses

Since the beginning of the tissue-engineering era, researchers have tried to develop materials with uniform microscale or nanoscale structures. Although these can induce the cell migration and proliferation required for tissue repair, their efficiencies were quite inferior compared to those of scaffolds with hierarchical structures that consist of both microsize and nanosize structures, as shown in **Figure 7**A.[210] In fact, overall cellular activities, including cell migration, orientation, proliferation, and differentiation, have been shown to be dramatically enhanced when scaffolds fabricated with a hierarchical structure are used. One reason for that better performance is the effective distribution of the stress over a large area, thus increasing the scaffold's toughness in general.[211] Moreover, the hierarchical organization has been adopted in nature by many species to

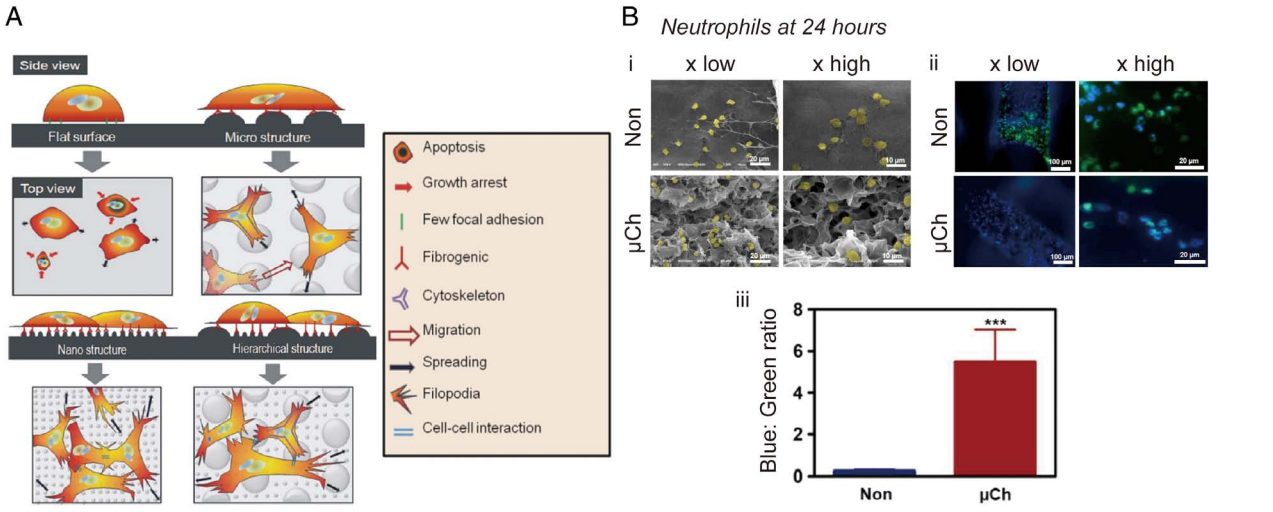

Figure 7. A) Schematic illustration of the effect of surface topography (flat, micro, nano, and hierarchical) on cellular responses. Reproduced with permission.[210] Copyright 2014, Wiley-VCH. B) Increased immune responses of cells in a hierarchical microchannel scaffold in vivo: i) SEM images of cells (yellow) attached to scaffolds at 24 h, ii) fluorescence images of extracellular DNA stained with SYTOX (green) and nuclei stained with DAPI (blue), and iii) ratio of blue/green fluorescence intensities from image (ii). Reproduced with permission.[215] Copyright 2020, Elsevier.

prevent the accumulation of somatic mutations.[212] This emphasizes the influence of hierarchical-based scaffolds on cell proliferation and differentiation during tissue healing.

Despite all the benefits of hierarchical structures, their manufacture is still a challenge to tissue engineers.[213] One must have a fair amount of knowledge about the structure of the target tissue to be able to mimic its hierarchical design from the molecular to the macroscopic level. A common example is the structure of bone in which nanoscale attributes affect cell binding, microscale components affect cell migration, and macroscale structures affect the mechanical anisotropy of the bone.[214] One method to enhance the hierarchical structure to mimic the Haversian canals in bone is to use electrophoretic deposition of a positive replica of chitosan/bioactive-glass scaffolds to create similar micropores, thus augmenting osteoconduction.[213] Taking a further step based on recent technical advancements, researchers have successfully manufactured hierarchically structured 3D-printed scaffolds called "μCh."[215] The interconnected 3D hierarchical structure in the microchannels can provide more space for efficient nutrient transport and metabolic waste disposal, thus enhancing osteogenesis.[216] Moreover, hierarchical structures with appropriate micropatterns can efficiently manipulate macrophage response by either increasing or reducing the macrophage polarization, thus altering the osteogenic differentiation of human bone marrow stromal cells.[217] These special scaffolds were also reported to enhance bone regeneration significantly in an early stage by reducing the formation of neutrophils (Figure 7Bi) while increasing cell survival as well as proliferation within the wounded tissue in an early stage (Figure 7Bii–iii).[215] This is a step closer to reproducing the natural bone–tissue structure and even further enhancing its functional activities.

Other targets of scaffolds based on hierarchical structures have also been extensively researched in recent years. Previous studies showed that hierarchical micro/nanofibrous structures can weaken the differentiation of fibroblasts into myofibroblasts, decreasing the excessive formation of the ECM and promoting the production of a continuous neodura tissue with characteristics similar to those of native skin tissue.[218] Tissue engineering of skeletal muscles has also achieved good results by incorporating micro/nanofibers into the hierarchical scaffolds. Such a structure was shown to encourage fiber alignment by affecting the expression of various myogenic genes, such as MyoD, myogenin, and troponin T, leading to increased myoblast proliferation and myotube formation.[219] Stem-cell engineering has also had noticeable success in creating microporous chitin microspheres (PCMSs) that contain interconnected nanopores, micropores, and macropores in a hierarchical porous structure, thereby creating interconnected nanofibril networks to retain the integrity of the PCMS. This leads to a remarkably improved proliferation and adhesion of human embryonic stem cells.[217]

6. Implementation of Hierarchical Tissue Constructs

Tissue engineering has been continuously developing over the past decades. Technical advances, as well as knowledge from related fields, such as materials science and engineering,[220] 3D printing technology,[18] nanotechnology, and cellular and developmental biology,[221,222] have been assimilated and have led to the promising implementation of both artificial tissue for drug development and disease modeling and personalized tissue engineering therapies in regenerative medicine. However, one of the common obstacles in developing and using artificial tissue is the current lack of ability to mimic the complexity of the tissue's structure and its natural microenvironment.[223–225] Therefore, in this article, we discussed recent developments and applications of fabricated artificial tissues with a hierarchical concept for regenerative medicine, including its utilization in pharmaceutical and pathophysiological research.

The inability of a 2D monolayer cell culture to resemble accurately and reliably a disease model has led to the emergence of microfluidic cell culture systems. The incorporation of a 2D cell culture into a microfluidic device has led to 3D approaches with sophisticated designs that mimic the mechanical and dynamic environments of native tissue.[226] Fabrication of complex, anisotropic, hierarchical macrostructures has been achieved by combining and integrating different technologies and materials. Bahmaee et al. used a fabrication method based on a polymerized high-internal-phase emulsion (polyHIPE) to advance an osteogenesis-on-a-chip device that incorporated a 3D environment and fluid shear stresses.[227] The channels inside the polyHIPE introduce a multiscale porosity in the device, which due to the hierarchical and interconnected porosity of polyHIPE, can support proliferation, differentiation, and ECM production.

As an implant, artificial tissue is continuously or sporadically in contact with body tissue or fluids to replace and restore the function of the native tissue that has been damaged. An implantable, multilayered (epidermis to dermis), and vascularized bioengineered skin graft for use with nonhealing cutaneous ulcers was fabricated using 3D bioprinting[228] and showed a vascular bed self-assembled from human endothelial cells with or without human placental pericytes in the dermal layer. Vascularization in a perfused implant is a sign of an artificial tissue that can adapt to its surrounding environment because the impregnated cells are supplied with nutrients until full in-growth of functional blood vessels from the surrounding host tissue is achieved.[229] Comparably, Kim et al. printed a vascularized skin patch from porcine-skin-derived dECM on immunodeficient mice by using both the extrusion and the inkjet printing methods.[230] Moreover, Cui et al. developed a cardiac patch with a 4D physiologically adaptable design, which included hierarchical macro and microstructural transformations tuned to the dynamic, mechanical process of a beating heart.[231] Recently, the first in-human cardiac bioimplant, named *PeriCord*, was implanted as an advanced-therapy medicinal product in injured myocardium clinical trials.[232] The bioimplant incorporates Wharton's jelly-MSCs from the umbilical cord as the active ingredient and human decellularized, lyophilized, and sterilized pericardium as the supporting cell material (vehicle) for surgical implantation. Decellularized tissues or organs contain natural hierarchical nano, micro, and macrostructures of endogenous tissues that do not require further biofunctionalization.[233] Differently from the implant constructed using a bioprinting process, decellularized matrices represent the closest constructs to natural ones.

7. Present Challenges and Perspectives

Ideally, the 3D microarchitecture and the mechanical and biochemical cues of tissue of interest have to be emulated in the fabrication of tissue-engineered constructs if the implant is to be integrated structurally and functionally in the body.[234] Among the preparation methods to set up a biomaterial-based scaffold, microfluidics and 3D bioprinting technology excel due to their precision, controllability, and versatility in preparing personalized biomaterials and complex tissues/organs. Advances in 3D printing have increased the feasibility of fabricating hierarchical tissues. For that reason, optimizing the microarchitectures of biopolymers and improving the efficacy of bioprinting are areas of active research. Furthermore, hydrogels and biopolymers that are biocompatible may not always be suitable for use with conventional bioprinting methods,[235] and the delivery of a particular number of cells may not be consistent for many tissue types.[236] One possible approach to addressing these issues involves combining substances to maximize the utility of each and to yield mechanical properties that can maintain the cell's functions under the mechanical and physical forces imposed by the printing and the postprinting processes.

Similarly, the identification of alternative materials for OOC devices is emerging as an important area of research, and PDMS is becoming the most commonly used material in microfluidic systems; however, it has drawbacks as the resultant film is thicker than the in vivo morphology, the absorbance changes of small hydrophobic molecules influence the efficacy of the solvent, and the material is toxic.[58] Hierarchical construct approaches in microfluidic technologies can provide the minimal function of a native tissue or organ to transform pharmaceutical preclinical examination by increasing throughput while minimizing the ethical and the financial concerns associated with in vivo assessment. The 3D bioprinting fabrication process for creating the complex structure of a natural organ and/or tissue and the microfluidic system to provide a microenvironment for the cell are the critical points in successful hierarchical tissue engineering. Accordingly, an adaptable microenvironment through the selection and the modification of suitable biomaterials is needed to modulate the behaviors of cells during tissue regeneration.[11]

For the purpose of constructing a hierarchical tissue in regenerative medicine, a comprehensive understanding of the materials, the fabrication method, the microenvironment, and the interactions between cells and scaffolds is fundamental. Moreover, the scaffold's mechanical properties, such as stiffness, geometry, and topography, which can induce biophysical responses, need to be considered. In addition, the material's degradation time and the controlled release of biochemical signaling molecules can affect the artificial tissue's cellular response. Thus, efforts to optimize the biomaterial's utilization, the microarchitecture of the artificial tissue, and the efficacy of the fabrication method are crucial for achieving more practical, hierarchical, adaptable, integrated scaffold–cell constructs for medical applications.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biofabrication, cell responses, hierarchical tissues, tissue engineering

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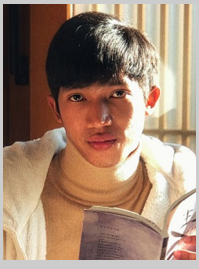

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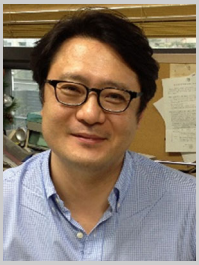

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