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Physically Active Bioreactors for Tissue Engineering Applications

N. Castro, S. Ribeiro, M. M. Fernandes, C. Ribeiro, V. Cardoso, V. Correia, R. Minguez, and S. Lanceros-Mendez**

Tissue engineering (TE) is a strongly expanding research area. TE approaches require biocompatible scaffolds, cells, and different applied stimuli, which altogether mimic the natural tissue microenvironment. Also, the extracellular matrix serves as a structural base for cells and as a source of growth factors and biophysical cues. The 3D characteristics of the microenvironment is one of the most recognized key factors for obtaining specific cell responses in vivo, being the physical cues increasingly investigated. Supporting those advances is the progress of smart and multifunctional materials design, whose properties improve the cell behavior control through the possibility of providing specific chemical and physical stimuli to the cellular environment. In this sense, a varying set of bioreactors that properly stimulate those materials and cells in vitro, creating an appropriate biomimetic microenvironment, is developed to obtain active bioreactors. This review provides a comprehensive overview on the important microenvironments of different cells and tissues, the smart materials type used for providing such microenvironments and the specific bioreactor technologies that allow subjecting the cells/tissues to the required biomimetic biochemical and biophysical cues. Further, it is shown that microfluidic bioreactors represent a growing and interesting field that hold great promise for achieving suitable TE strategies.

1. Introduction

Tissue engineering (TE) is a dynamic and growing scientific field that merges the knowledge of different areas such as biology, physics, medicine, and engineering.[1] The TE discipline was first coined at a National Science Foundation sponsored meeting in 1987, thus originating a field that employs life sciences and engineering with the purpose of developing biologic or synthetic supports to restore, keep, or enhance tissue functions or damaged organs.[2]

The classical TE approach has been mainly focusing on associating cells with a supporting matrix, also called biomaterials or scaffolds, that essentially acts as a passive template for tissue formation in vitro by allowing cells to adhere, migrate, differentiate, and produce tissue. Usually, the cells are seeded onto the scaffolds and, occasionally, growth factors are also added. The combination of cells, growth factors, and scaffold is often referred as the TE triad.[3] Nevertheless, novel approaches in TE that include the application of a biophysical stimuli to the scaffolds through the use of a bioreactor are emerging.[4]

N. Castro, Prof S. Lanceros-Mendez
BCMaterials, Basque Centre for Materials, Applications and Nanostructures
University of the Basque Country UPV/EHU Science Park
Leioa E-48940, Spain
E-mail: senentxu.lanceros@bcmaterials.net

Dr. S. Ribeiro, Dr. M. M. Fernandes, Dr. C. Ribeiro
Physics Centre
University of Minho
Campus de Gualtar, Braga 4710-057, Portugal
E-mail: sribeiro@fisica.uminho.pt

Dr. S. Ribeiro
Centre of Molecular and Environmental Biology (CBMA)
University of Minho
Campus de Gualtar, Braga 4710-057, Portugal

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Dr. M. M. Fernandes, Dr. C. Ribeiro
CEB – Centre of Biological Engineering
University of Minho
Braga 4710-057, Portugal

Dr. V. Cardoso
CMEMS-UMinho
Universidade do Minho
Campus de Azurém, Guimarães 4800-058, Portugal

Dr. V. Correia
Algoritmi Research Centre
University of Minho
Campus de Azurém, Guimarães 4800-058, Portugal

Prof. R. Minguez
Department of Graphic Design and Engineering Projects
University of the Basque Country UPV/EHU
Bilbao E-48013, Spain

Prof. S. Lanceros-Mendez
IKERBASQUE
Basque Foundation for Science
Bilbao E-48013, Spain

In TE, it is important to select appropriate components for the tissue under study, e.g., the type of cells (the main base for tissue culture as they contain the preprogrammed information that allows tissue regeneration), the type of biomaterial (their structural and mechanical properties) and the culture format. It is also important to properly assess their interplay in order to develop optimized platforms for tissue regeneration.[4]

Mimicking the natural microenvironment of tissues with engineered scaffolds remains one of the greatest challenges in TE. The envisaged microenvironment is a complex 3D microstructure comprising signaling molecules, cells, and structural components. Each one of these components plays a critical role in healthy tissue, thus it is very important to understand their interactions to further identify the proper methods to repair damaged tissues. Furthermore, by understanding the contribution of the tissue microenvironment in cell fate determination, a more accurate and specifically designed TE strategies may be delineated.[5]

In order to tailor cellular behavior and function, or even tissue development, repair and/or regeneration, multiple physiological clues are required. Besides the well-reported biochemical cues, physical signals such as electrical and mechanical ones are known to act synergistically to strengthen their outcome, and thus are being increasingly investigated in regenerative medicine.[6]

In fact, the knowledge that cells and tissues feel the surrounding environment, being able to translate physical stimuli into biochemical and biological responses, has been paving the way for the development of smart materials to be applied in regenerative medicine.[7] These materials actively contribute to cell and tissue regeneration and do not just act passively for cell adhesion, growth, and proliferation.[8] They are indeed smart biomaterials that participate on the process of tissue regeneration. But how to make them smart? These biomaterials, which may be developed in a variety of designs,[9] are constituted by some specific components that respond to physical or chemical stimuli by providing different biophysical cues, present in vivo, and that are typically provided by specific bioreactors in vitro.[10] The response of these materials to the bioreactor-induced stimuli, is given in such a way that creates a microenvironment that resembles the native microenvironments of tissue development, which allows them to actively participate on the regeneration mechanisms, making these materials smart. This is the concept of active bioreactors.

Thus, bioreactors and smart materials enable simulating in vitro an in vivo state environment in order to understand normal cell/molecular physiology; develop cells for medical purposes; gene therapies or pathological state simulations for disease studies and progress parameters. They allow to test new potential treatments for new therapeutic strategies in more realistic conditions in comparison with conventional static in vitro cultures.[11] Nevertheless, bioreactors are used not only for medical purposes, but have also been employed in fields such as fermentation, water treatments, food processing, and pharmaceutical products.[12] The main reasons for their use are their working principles based in mimicking biologic and biochemical processes, aiming for a strict monitored and controlled environment. Over the last few years, the bioreactors have become increasingly needed. Due to limited understanding of specific physicochemical culture parameters regulatory role on tissue development, the need for bioreactor systems with reproducible and controlled variations of a specific environment in an in vitro culture has become key in this multidisciplinary engineering field. These systems provide the required technology to expose cell function vital mechanisms while enabling a potential improvement in engineered tissues quality, by increasing mass transport inside 3D structures with higher automation.[13] According to bioreactor type, static or dynamic cultures or both are allowed, where static cultures mainly rely in providing a setup support for cells seeded in scaffolds with medium. However, the dynamic culture provides that as well as time-dependent stimuli, which improve cell proliferation and differentiation. Stimuli types play an important role depending on the specific type of cells in a TE culture. For that, several bioreactor setups (commercial and custom built) according to production capacity and use (from mass production to laboratory scale) can be found in the literature.[14] In addition, using these setups enable learning of standard control culture parameters of different tissues types, allowing the corresponding standardization for future automated production processes. These may be designed in order to both, reduce production costs and widespread the use of engineered tissues, increasing applicability and reaching a next phase of further research, such as 3D organ printing. Furthermore, most bioreactors can be classified into macrobioreactors that are primarily use to grow functional tissues for in vivo implantation, and microbioreactors, which are used to develop native tissue-like environment for drug testing and other in vitro studies on biochemical and mechanical regulation of cell responses and tissue development.[15] Ultimately, the advances achieved in 3D printing techniques have open important opportunities for advances in regenerative medicine, once this technique can produce scaffolds with a high degree of complexity and precision, allowing to provide detailed biomimetic 3D structures.[16] In this way, it is expected that TE allied to printing technologies will allow culture conditions that may be used for growing fully functional and viable organs for transplantation in vivo.[17]

2. Relevance of Cell Microenvironments

Biological tissues are complex organisms where cellular "building blocks" cooperate to provide tissue-specific functions. These "blocks" are an ensemble of cells and molecules, encased in an ECM that together form a biological system with a specific role, establishing an intricate microenvironment.[18] The complexity of these microenvironments makes it difficult to mimic all the conditions in vitro. In most of the cell-culture studies performed in vitro, the cells are grown in 2D platforms and interact with proteins that naturally adsorb to their surface. However, 2D cell-culture environments have some limitations, including limited cell–cell interactions when compared to 3D environments, a low degree of control over cell–adhesion interactions and signaling, and the limitations (e.g., in terms of mechanical properties) of the materials commonly used for cell culture.[19] Therefore, novel approaches are increasingly being sought to develop in vitro culture models designed to control the behavior of multiple cells thus mimicking the cellular microenvironments

triggering specific cell functions. One factor that has been suggested to regulate cell survival and maintenance, proliferation, and differentiation is the 3D microenvironment where cells grow, the so called niche.[20] In fact, recent works have reported the growth of cells in 3D gels made of collagen or reconstituted ECM proteins that dramatically changed cellular responses when compared to observations of cell cultures on polystyrene surfaces.[19,21] The combination of 3D environment and the presence of ECM proteins in such a way that mimics the real in vivo environment has been regarded as a needed approach to develop TE strategies. The created microenvironment regulates cell functions through several mechanisms, being the direct contact between cells and their adjacent niche cells the best investigated one. Apart from this mechanism, the microenvironmental events that have been reported to coordinate the balance of cell self-renewal, proliferation, and differentiation are the cell–ECM interaction, mediated either by adherence or gap junctions, or the presence of soluble and immobilized factors, cytoskeleton and integrins (**Figure 1**).[22]

To trigger these interactions among the cells, microenvironments providing biochemical stimuli is the common approach to be applied. These include the delivery of exogenous growth factors and hormones,[23] cell-adhesive peptides,[24] and membrane-attached receptors ephrins.[25] Nevertheless, the lack of availability of required growth factors and peptides, insufficient bioactivity in vivo, difficult control over dose administration and off-target delivery, often limit their clinical potential.[26] This issue has prompted an opportunity in the search for physical stimulatory alternatives (such as electrical, mechanical, thermal, and magnetic).[27] Despite their ability to influence cell migration, orientation, proliferation, and differentiation, these kinds of stimuli have been poorly explored in TE. In fact, the effect of physical factors on the alteration of the extracellular environment for triggering the activation of intracellular signaling cascades and gene expressions in a cell, has demonstrated to be fundamental for TE.[28] Cells and tissues are indeed able to sense physical stimuli and translate them into biochemical and biological responses. Thus, the proper in vitro microenvironment should comprise both physical and biochemical factors apart from the important 3D morphology. The physical-mediated processes in a cell and living tissues include different phenomenon such as: i) mechanotransduction, when activated by a mechanical stress, ii) thermotransduction, when activated by temperature, iii) electrotransduction, when activated by an electrical field, and iv) magnetotransduction, when activated by a magnetic field.

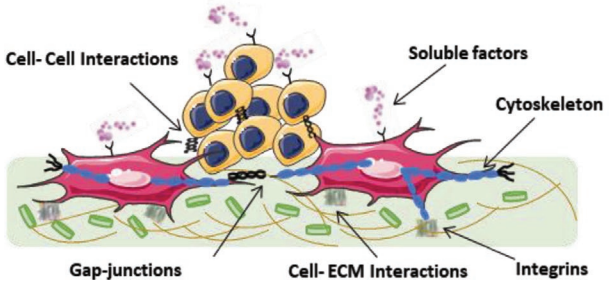

Figure 1. Schematic representation of microenvironmental events that coordinate the balance of cell self-renewal and differentiation into functional mature cells. Reproduced with permission.[22] Copyright 2010, Wiley-VCH.

2.1. Cellular Mechanotransduction

Mechanochemical transduction or mechanotransduction is the process of converting mechanical forces into biochemical activities and gene expression in cells.[29] Understanding mechanotransduction at cellular and molecular level has been a valuable tool for the development of strategies to be applied in regenerative medicine.[30] The simple observations and common knowledge that mechanical loading induces the strengthening of skeletal muscles, tendons, ligaments, and bone[31] while the prolonged exposure of early astronauts to weightlessness environments made their bones prone to fractures,[32] led to the conclusions that phenomenon of cellular mechanotransduction plays an important role on human body. Although with a negative outcome for health, the same effect is observed in the heart due to hypertension where the blood pressure overload results in a hypertrophic thickening of the tissue.[33] The observation of these events was the primary motivation for the study of mechanotransduction in cells and tissues. In a broader sense, the musculoskeletal system of human body is constantly subjected to external forces such as gravity and exercise-induced stress that provide mechanical stress to the whole body. But cells and subcellular structures are also exposed to different forces. These include the shear-stress of blood flowing across endothelium, the microscopic forces that cells induce on the surrounding ECM and on each other or the stretch of vessels owing to blood pressure (**Figure 2**).[34] These different endogenous forces further trigger specific cell functions.[30] The detailed mechanisms by which cells respond to mechanical stimuli and transfer the forces to the mechanotransducers have been well reviewed by Schwartz et al..[35] The magnitudes of the forces vary among different cell types, which also exhibit different sensitivities to the frequency, magnitude, and duration of the forces. Inside the cytoplasm, forces of a few picoNewtons (≈pNs), may be generated. Nonmuscle myosin II are particularly interesting "molecular motors" because these molecules can align along actin microfilaments to significantly increase the total magnitude of forces in actin bundles to the nanoNewton ((≈nN) range at a single focal adhesion.[36] Forces from a few pNs to several dozens of pNs are estimated to be exerted at single integrins in cell.[36–37] For example, a single integrin molecule has been reported to generate a ≈40 pN force to initiate cell spreading before the formation of focal adhesions[38] and that a >54 pN molecular tension is transmitted by clustered integrins in motile actomyosin-dependent focal adhesions.[39]

2.2. Cellular Electrotransduction

It is well known that human body experiences and generates biological electric field and current.[30] Back in 1983, Barker et al. measured the electrical potentials in various body locations, which ranged from 10 and 60 mV, focusing on the "battery" existing in the skin at epidermis and its possible role in wound healing.[40] The biological effect of electric field was

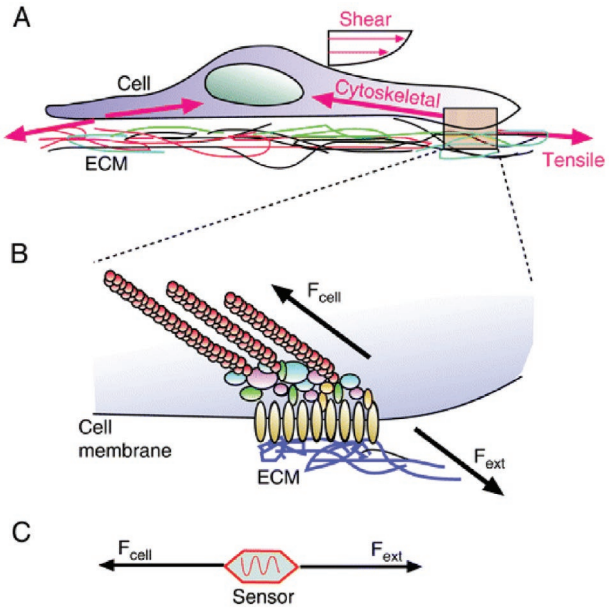

Figure 2. Schematic representation of the mechanical stress that cells are exposed to. A) Representation of the shear forces through fluid flow over the cell, tensile forces acting through the ECM, and cytoskeletally generated contractile forces. Depicted is a single cell attached to a complex ECM (illustrated as a multicolored fabric). B) Close-up of a focal adhesion showing the balance of external and internal forces in driving stress at a mechanosensor. Depicted are actin stress fibers (red) anchored into focal adhesions (multicolored array of proteins) that bind to the ECM (blue) through integrins (brown). C) This balance of forces provides the stress necessary for mechanical sensing. Reproduced with permission.[34] Copyright 2008, The Company of Biologists.

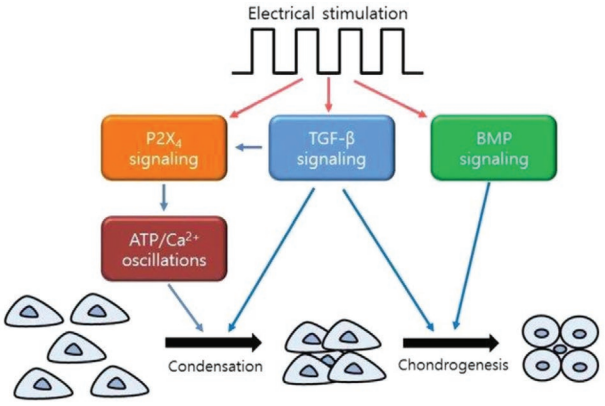

Figure 3. Proposed model of the functions of electrotransduction for MSC chondrogenesis. ES drives ATP/ Ca^{2+} oscillations, leading to MSC condensation through TGF-β signaling and P2 × 4 signaling, and subsequently induces chondrogenesis through TGF-β signaling, BMP signaling and P2 × 4 signaling. Reproduced with permission.[52] Copyright 2016, Nature.

also later extensively reviewed by Zipse.[41] That is why the process of cellular electrotransduction is the one of the most investigated processes. In fact, living cells and tissues show many properties that are based or triggered by electrical signals. As an example, our sense of touch is transmitted to the brain via electrical pulses.[42] Across the plasma membrane, an electrical voltage is also present as inside the cell membrane the environment remains more negatively charged than the outside.[43] Indeed, the cell microenvironment closely resembles an electrical system. Cells generate electromotive force, use varying resistances in series or in parallel, regulate the potential differences whenever needed, switch on and off, control and rectify current flow and store charge. Due to all these properties, the application of small electric fields is known to guide the development and regeneration of many tissues. Such examples are the epithelial[44] and corneal[45] cells that has been described to be guided by electrical fields, as well to induce them to move and migrate in cell culture. Vascular endothelial cells have been modulated by electric fields[46] while an enhanced nerve fiber outgrowth in vitro[47] was observed. Also, a topic that has been widely explored is the application of small electric fields to bones and cartilage to promote regeneration and healing.[48] Some of the strategies take advantage on the fact that bone is piezoelectric and develop varying electrical charges on its surface when subjected to a mechanical force.[49] Since walking, running, or jumping provides such stimuli, the use of piezoelectric materials has been calling great attention from the scientific community.[50] Many theories suggest that cell membrane is the main target of the application of electric field, owed to its well-known bioelectrical properties. It has been suggested that electrical fields are combined with internal signaling pathways via interactions with sensor mechanisms located at the cell membrane inducing an electrotransduction process.[51] Electrical stimulation (ES) has been found to induce different signaling processes. Chun et al. have shown that electrostimulation induce Ca^{2+}/adenosine triphosphate (ATP) oscillations, leading to mesenchymal stem cells (MSCs) chondrogenesis, also showing that P2 × 4 signaling mediates ES-driven ATP oscillations and chondrogenesis, and transforming growth factor- β (TGF-β) and bone morphogenic protein (BMP) signaling both mediates ES-driven chondrogenesis but have differential effects on ES-driven condensation (**Figure 3**).[52] The proven effect of electrostimulation on cell, in a biomimetic approach, has been paving the way for the development of novel technologies for cell regeneration based on the processes of electrotransduction.

2.3. Cellular Thermotransduction

The sensitivity of cells to thermal changes is widely known and has been used to develop strategies for hyperthermia to kill cancer cells.[53] High temperatures cause direct injury to cancerous cells and sensitize the cells to other treatment modalities, with little injury to normal tissues.[54] However, before being used as a possibility to treat cancer, the thermal sensitivity of cells have been studied to promote different cellular functions. Heat was found to induce the activation and sensitization of pain-sensitive neurons and the possibility to reduce pain by the use of protein kinase C has been suggested.[55] The simple and widely known fact that thermosensory neurons are able to detect temperatures warmer and cooler than optimal temperatures, give signs for body temperature change thus avoiding tissue-damaging by heat. It has been suggested that

cells sense the temperature via channel activation in mammalian neurons.[56] Pulses of heat induce the release of Ca^{2+} from intercellular compartments and thus heat-sensitive current could be inhibited by a of Ca^{2+} chelator.[57] On the other hand, sensing the cold are controlled by specific thermoreceptor nerve endings, in which the modulation of the Na^+–K^+ ATPase,[58] differential temperature sensitivity of Na^+ and K^+ channels and closure of K^+ channels[59] play an essential role. Nevertheless, the mechanisms mediating the cold sensing remain poorly defined.

2.4. Cellular Magnetotransduction

The effect of magnetic fields on cellular response has been less studied than mechanotransduction processes since no "magneto-receptors" have been identified in cells.[60] Nevertheless, evidences have been showing that magnetic fields targets the plasma membrane in cells, owed to its bioelectrical properties.[61] Theories suggest that the cell membrane is altered by a pulsed electromagnetic field, namely the embedded ion channels, ligand-binding sites, and the density and distribution of receptors, which further induces transmembrane signaling cascades.[62] Other studies have also proposed that the magnetic field affects the ion/ligand-binding kinetics[63] or the cyclotron resonance (i.e., the movement of ions in a magnetic field) in membrane transport.[64] Evidences have shown that the application of magnetic fields, alone or in combination with static field affect the Ca^{2+} signaling cascade, resulting in an increased content intracellularly.[65] Rubio et al. have recently reached the same conclusion, showing that spatiotemporal magnetic field variations induced an increase on the Ca^{2+} levels in cytosol as well on the actin polymerization, which is mainly due to the activation of voltage-gated sodium channels[66] (**Figure 4**).

It has been demonstrated that static magnetic fields does not have a significant effect on the basic properties of cell growth and survival under normal culture conditions,[67] thus the magnetic stimulation of cells has been valuable to obtain remote control of cellular functions. Only a few tools are currently capable of manipulating cellular events at distance, at spatial and temporal scales matching their naturally active range.[68] Therefore, the use of magnetic fields together with materials comprising magnetic nanoparticles for remote cell stimulation is a promising approach. Indirectly, magnetic field may induce other processes of mechanotransduction, thermotransduction, or electrotransduction remotely by triggering molecular stimuli via mechanical, thermal or biochemical perturbations.[69] This has been valuable for the development of novel smart materials that respond to magnetic stimuli or magnetic responsive materials to be applied in regenerative medicine.[69,70] Magnetic materials possess important properties that have allowed applications in different medical areas including neurology,[71] ophthalmology,[72] dentistry,[73] and cardiology,[74] among others. In fact, the size of these magnetic nanomaterials has been playing a key role, imparting unique properties to the material. Specific interactions with cells, viruses, and proteins, which ultimately induce cellular growth or death, and the possibility of entering the body and reach spaces that are inaccessible by other materials,[75] are just few examples of the important properties these materials hold. Moreover, at sizes below 20 nm, magnetic nanoparticles exhibit superparamagnetic behavior and no remnant magnetization is observed when the magnetic field is removed, making them suitable for in vivo applications since it prevents aggregation and enables to easily redisperse rapidly after withdrawing the magnetic field.[75a] Also, one of the advantages of using magnetic nanomaterials in biomedicine is the fact that they are easily traceable and localized inside the body through the action of a magnetic field, thus using minimally invasive methods.[76] Mimicking cell microenvironments is thus very important to replicate in vitro what occurs in vivo. Strategies that include providing the right biochemical stimuli combined with 3D morphologies and taking advantage on the sensitivity of cells to mechanical, electric, thermal, and magnetic cues is must be included in the future efforts of tissues engineering and regeneration. Therefore, the application of stimuli to induce mechanical, thermal, electric and magneto-transduction processes holds great promise, since it actively responds to a biomimetic stimulus that control processes of cells regeneration and homeostasis.

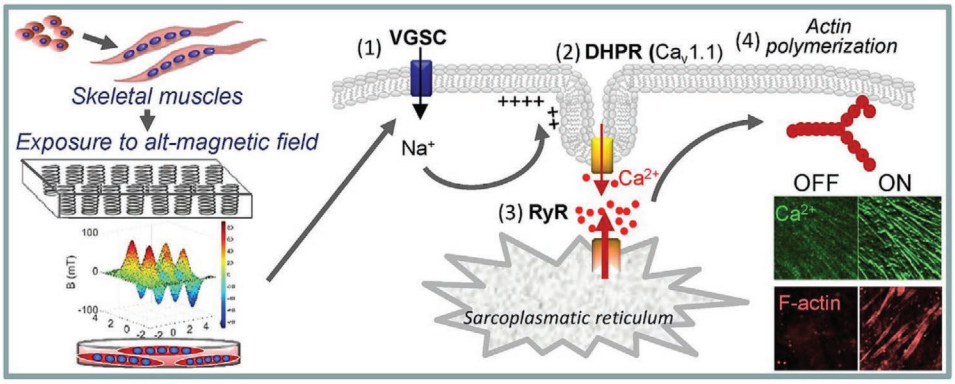

Figure 4. Schematic representation of the spatiotemporal magnetic field variations that enhance cytosolic Ca^{2+} levels and induce actin polymerization via activation of voltage-gated sodium channels in skeletal muscle cells. Reproduced with permission.[66] Copyright 2016, Elsevier.

3. Suitable Materials and Stimuli for Smart Scaffolds Development

Multifunctional biomaterials based on smart materials have been applied in several TE fields, which include bone, cartilage, muscle and neural regeneration, cardiovascular and wound healing, and also skin growth. Depending on the area of application, the biomaterial requirements are different. The microenvironment found in each tissue or organ can be highly distinct, from the morphology to the stimuli they are subjected in vivo. For example, in the case of the bone, the microenvironment is described as a complex structural and biological system, constituted by three phases: the organic phase (mainly collagen), the mineral phase (mainly crystalline hydroxyapatite), and pores.[50f] Also, in the human adult, it is composed by 80% of cortical bone and 20% of trabecular bone, being these ratio dependent on which bones and skeletal sites we are dealing with.[77] Another important characteristic of the bone is its piezoelectric nature,[50b] whose properties have been regarded as responsible for bone regeneration, involving electromechanical processes: when the bone is mechanically stressed, electrical signals are produced which in turn promote bone growth and remodeling.[50b]

Regarding the skeletal muscle, it is considered a highly adapted tissue, established by long parallel bundles of multinucleated contractile muscle cells, the myofibers.[50a] Besides that, these cells are embedded in a 3D tissue undergoing mechanical stretch and compression and also the skeletal muscle cells develop endogenous electrical fields in the form of membrane potentials.[78] Contrarily, endothelial cells are found in a 2D interface in contact with the fluid, exposed also to stretch and compression during pulsatile blood flow and to fluid shear stress.[79]

The neural tissue is the main tissue component of the nervous system, that is divided in two main categories: neurons and neuroglia.[80] Also, the neural tissue is a complex 3D environment with different topographical features, with a large range morphologies and size scales.[81] During neural development, local electric fields are measured, being bioelectrical control mechanisms relevant for the development and regeneration of nerve fibers.[82] In this way, for better and more efficient TE approaches, the platforms design must take into consideration the mechanobiological and electrobiological niche of each tissue. It is important to re-emphasize that the development, repair and/or regeneration of tissues and organs and also the cell behavior and function, require multiples physiological clues, not only the (bio)chemical ones, but also the physical signals, namely the electrical and mechanical ones.[6,83] So, in order to achieve the requirement for proper tissue regeneration, different kinds of materials and morphologies must be used. Regarding the literature, it has been verified that biomaterials with smart properties in different forms, ranging from films, fibers, porous 3D scaffolds to patterned surfaces, have been employed depending on the TE specific area. Also, 3D printed scaffolds have emerged as a new tool for the development of functional and smart scaffolds in order to provide a suitable environment for cell growth.[84] These biomaterials have been used with different bioreactors that can provide different stimuli, being the mechanical, electrical, and magnetic ones the most used stimuli. The different kind of materials, morphologies and stimuli applied for the different TE areas are summarized in **Table 1**. In bone TE, the three stimuli have been employed, but predominately mechanical and electrical ones. Relatively to the morphology, films, and fibers have been mainly used. For mechanical stimuli, piezoelectric materials have been chosen and for magnetic stimuli also with the incorporation of magnetostrictive particles or hydroxyapatite. For example, piezoelectric films, namely polyvinylidene fluoride (PVDF), have demonstrated in dynamic conditions to provide an adequate environment for enhancing the growth and differentiation of goat marrow cells into osteoblast, exhibiting an ideal support for the seeding and the development of undifferentiated cells toward a desired phenotype.[85] Also, when these kind of physical stimuli are combined with biochemical ones, this leads to enhanced osteogenic differentiation of human adipose stem cells, demonstrating a successful approach of the biomimetic microenvironment present in the human body.[86] Also, collagen type I has been exploited, demonstrating that when it is subjected to mechanical stretching, it is possible to promote the proliferation and differentiation of human osteoblastic precursor cells.[87] In a recent approach, magnetoelectric biomaterials have been developed, demonstrating bone cell proliferation under mechanical and electrical stimulation remotely triggered by the application of a varying magnetic field.[70b,88] Relatively to cartilage, piezoelectric materials have been also used (namely PVDF and collagen), but also polycaprolactone (PCL) and until now, only mechanical (compression) stimuli have been studied. Concerning the morphology, porous scaffolds, fibers, and hydrogels have been selected. By applying mechanical deformation, it has been demonstrated that piezoelectric fibrous scaffolds with low voltage output promoted chondrogenic differentiation contrary to those with high voltage output that promoted osteogenic differentiation.[89] Hydrogels have been also investigated under dynamic compressive strain, verifying an enhanced chondrogenic phenotype, where the mechanical stimulation promotes the adipose-derived stem cells differentiation toward the chondrogenic lineage.[90] In the skeletal and cardiac muscle TE area, once both are subjected to mechanically and electrically active microenvironments, mechanical and electrical stimuli have been employed. Different kind of materials and morphologies has been investigated, but namely aligned morphologies for both and also hydrogels for cardiac tissue. The topographical alignment of the biomaterials combined with ES has been investigated and it was found to be an effective tool to promote myogenic differentiation and maturation, providing the basis for therapeutic strategies for skeletal muscle regenerative TE.[91] The electromechanical cell conditioning of cardiac cells before delivery has demonstrated to be an promising alternative for heart repair.[92] Also, injectable hydrogels with tailored electrical properties submitted to an electrical stimuli demonstrated to be an promising injectable biomaterial for cardiac TE.[93] For neural regeneration, mechanical and electrical stimuli have been also used but mostly the electrical ones. Relatively to the material, the most used are piezoelectric and conductive materials; and different morphologies have been studied (films, fibers, scaffolds, and hydrogel) but mostly

Table 1. Biomaterial and stimuli applied for different TE applications.

Tissue	Biomaterial	Stimuli applied			Refs.
		Mechanical	Electrical	Magnetic	
Bone	β-PVDF film	Frequency of 1Hz	–	–	[50d]
		1 Hz with maximum amplitude of ≈1 mm	–	–	[86]
		A lab rotator (model DSR 2800 V, Digi-system Laboratory Instruments)	–	–	[85]
		–	Alternating sinusoidal current (AC), of 5 V, at 1 and 3 Hz for 15 min at each frequency	–	[103]
	Poly(vinylidenefluoride-*co*-trifluoroethylene) (P(VDF-TrFE)) nanofibers	Vibration with a frequency of 2 Hz and voltage of 4V	–	–	[104]
	P(VDF-TrFE)/ boron nitride nanotubes (BNNTs) film	Twice a day for 10 s	≈20-60 mV	–	[105]
	Multiwalled carbon nanotubes/ chitosan/β-glycerophosphate scaffold	–	1 V voltage, 50 Hz frequency and 5% duty cycle	–	[106]
	Collagen gels	Frequency of 1 Hz, 1% of strain and 1800 cycles	–	–	[87]
	3D collagen fibers	–	0.2, 0.4, and 0.7 V min^{-1} for 60 min	–	[107]
	3D collagen scaffold	–	Electrical stimulation chamber direct currents from an amplifier	–	[107]
	Self-doped sulfonated polyaniline-based interdigitated electrodes	–	500 mV, 1 kHz	–	[108]
	Polypyrrole (PPy)/PCL scaffold	–	200 µA (DC) for 4 h per day	–	[109]
	PPy films	–	0.35 V cm^{-1} for 4 h	–	[110]
	Poly-L-lactide acid (PLLA)/carbon nanotube (CNT) nonporous substrates	–	10 mA, 10 Hz (AC) for 6 h per day	–	[111]
	Polylactide acid (PLA)/CNTs nanofiber	–	100 µA (DC)	–	[112]
	Terfenol-D/PVDF-TrFe films	–	–	0.3 Hz, 20 mm displacement, 230 Oe–0 Oe, ME voltages up to 0.115 mV	[88]
	PVDF-cobalt ferrite (CFO) nanoparticles 3D scaffolds			0.3 Hz, 20 mm displacement, 230 Oe–0 Oe, ME voltages up to 0.115 mV	[70b]
	Hydroxyapatite disks	–	–	Magnetic field equal to 2 ± 0.2 mT, 5 ± 1 mV, 75 ± 2 Hz, pulse duration of 1.3 ms	[113]
		–	–	15 V DC, static magnetic field of 100 mT for 30 min every 2 d	[114]
Cartilage	Polycaprolactone–β- tricalcium phosphate (PCL-TCP) blended scaffolds	Cyclic compression at 0.22%, 1 Hz for 4 h per day; Biaxial rotation at 5 rpm and an angle of rotation at 90°	–	–	[115]
	PCL/fibrin scaffolds	30 min with onset strain of 15% at a frequency of 1 Hz, and 90 min of stillness	–	–	[116]
	Polyglycolic acid fibrous disks	Compression at sequential increments of 10% strain to a maximum of 40% strain	–	–	[117]
	PVDF-TrFE and PCL fibers	1 Hz with 10% deformation	–	–	[89]
	Collagen and agarose hydrogel	5% and 15% applied compression for 7 d. The compression phase of dynamic cyclic loading at a rate of 20 µm s^{-1}.	–	–	[118]

(*continued*)

Table 1. (Continued)

Tissue	Biomaterial	Stimuli applied			Refs.
		Mechanical	Electrical	Magnetic	
	Gelatin/chondroitin-6-sulfate/hyaluronan/chitosan highly elastic cryogels	1 Hz, 20% strain and 3 h per day for 14 d	–	–	[90]
Skeletal muscle	PCL aligned topography	–	Intermittent EF cycles 10 min separated by 20 min for 3 h	–	[91a]
	Fibrin, a natural hydrogel, scaffold	3 d after construct preparation, 6 h of static strain at 10% (exercise phase) followed by 18 h at 3% (rest phase) until 9 d	–	–	[119]
	Collagen/Matrigel	25% change in length. It consists of three sets of five stretch/relaxations followed by ≈28 min of rest. The strain amplitude was 5% strain for 2 d (days 8–10 after casting), 10% strain for 2 d (days 10–12 after casting), and 15% strain for 4 d (days 12–16 after casting)	–	–	[120]
Cardiac muscle	Collagen/fibrin 3D matrices	–	Biphasic pulses, 5 V cm^{-1}, 0.2 Hz, 1 ms	–	[121]
	Silicone-patterned surface	–	Monophasic square-wave pulses, 2 ms, 50 mV cm^{-1}, 1 Hz	–	[122]
	Fibrin hydrogel	10% stretching	2 ms pulses of 50 mV cm^{-1} at 1 Hz	–	[92]
	Electroactive tetraaniline-containing thermosensitive hydrogels	–	Square wave, frequency of 100 Hz, 50% duty cycle, and electrical potential of 0.5 V	–	[93]
Neural	Stable glycol-chitosan- BNNT	Ultrasound stimulation, 20 W, 40 kHz	–	–	[123]
	PVDF-TrFE/barium titanate film	Ultrasounds, 5 s twice a day	–	–	[124]
	Barium titanate nanoparticles	Ultrasounds, 0.1, 0.2, 0.4, and 0.8 W cm^{-2}	–	–	[125]
	PVDF film	50 Hz			[126]
	Poled PVDF fibers	–	2–3 mV, 1200 Hz	–	[47]
	PPy film	–	100 mV (DC)200	–	[127]
	PPy scaffold	–	Biphasic pulses, 100 µs, ±1 mA cm^{-2}, 250 Hz	–	[128]
	PPy/PLA nanofibers	–	0.1 V, 10 µA	–	[127]
		–	100 mV	–	[129]
		–	+1 V to –1 V square wave 1 kHz	–	[130]
		–	100 mV mm^{-1} (DC)	–	[131]
	PCL/PPy nanofibers	–	40 mV		[132]
	PPy/chitosan membrane	–	10 V (DC)	–	[133]
		–	100 mV (DC)	–	[134]
	PPy/ poly lactic-glycolic acid (PLGA) electrospun meshes	–	10 mV cm^{-1}	–	[135]
	PLGA films coated with PPy	–	10 mV cm^{-1}	–	[136]
	Poly (D,L-lactide-*co*-epsilon-caprolactone) (PDLLA/CL) membrane coated with PPy	–	0, 2, 8 and 20 µA mm^{-1} (DC)	–	[137]
	PPy/ poly lactic-glycolic acid (PLGA) electrospun meshes	–	10 mV cm^{-1}	–	[135]
	PLGA films coated with PPy	–	10 mV cm^{-1}	–	[136]
	Poly (D,L-lactide-*co*-epsilon-caprolactone) (PDLLA/CL) membrane coated with PPy	–	0, 2, 8, and 20 µA mm^{-1} (DC)	–	[137]

(*continued*)

Table 1. (Continued)

Tissue	Biomaterial	Stimuli applied			Refs.
		Mechanical	Electrical	Magnetic	
	Polyaniline (PANI) with PCL/gelatin nanofibers	–	100 mV (DC)	–	[138]
	PANI/poly(L-lactide-*co*-ε-caprolactone) (PLCL) nanofibers	–	0–200 m (DC)	–	[139]
	Copolymer of hydroxyl-capped PLA and carboxyl-capped aniline pentamer (AP) film	–	0.1 V, 1 Hz	–	[140]
	Sodium alginate hydrogel	–	Frequencies of 0.1, 0.5, 1 and 10 Hz Field amplitudes of 2, 4, and ≈16 V m^{-1}	–	[141]
	PEDOT:PSS film	–	Pulsed stimulation potential, frequency of 100 Hz	–	[142]
	Graphene–poly(3,4-ethylenedioxythiophene) hybrid microfibers	–	3000 pulses per day, frequency of about 120 times per min	–	[143]
	PLLA/PANI nanofibrous scaffolds	–	100 mV mm^{-1}	–	[94]
Skin	PPy/PLLA membranes	–	100 mV mm^{-1} (DC)	–	[144]
	PPy/PDLLA membranes	–	100 mV (DC)	–	[145]
	PPy/PLLA membranes	–	0.05 V mm^{-1} (DC)	–	[96]
Vascular	PANI-coated PCL fibers	–	400 mV cm^{-1}, 30 min per day	–	[146]
	Collagen gels	Static equibiaxial stretching (10%), cyclic equibiaxial stretching (7% and 20%), and cyclic equibiaxial stretching with incrementally increasing stain magnitude (7% → 15% → 20%)	–	–	[98]
Wound healing	Polyurethane/PVDF fibers	Dynamic deformation of 8% at 0.5 Hz for 24 h	–	–	[100]
	PPy on the surface of polyethylene terephthalate (PET) substrates	–	100 mV mm^{-1}, 10 s stimulation within a period of 1200 s or 300 s stimulation within a period of 600 s	–	[99]
Tendon	Collagen scaffolds	3% preload, a 10% deformation, and a stimulation frequency rate set at 0.5, 1, and 2 Hz which alternates stimulation/resting phases for 7 and 14 d	–	–	[102]
	Nanofibrous PCL	Cyclic uniaxial strain with 4% elongation along the direction of PCL nanofiber yarns in the woven fabrics and a frequency of 0.5 Hz for 2 h per d during 12 d.	–	–	[101]

aligned fibers. It was demonstrated that when the piezoelectric biomaterials were electrically stimulated, higher nerve regeneration was observed compared to the nonstimulated scaffolds.[47,94] In skin and vascular TE, few studies with dynamic conditions are performed with only electrical stimuli. For both applications, composites with conductive materials have been used, being that the morphology used is different in both cases. Membranes have been selected for skin regeneration whereas randomly oriented fibers have been selected for vascular tissue. The application of electrical stimuli has demonstrated to be an effective approach to upregulate the mitochondrial activity of human skin fibroblasts[95] but also to regulate tissue regeneration in conductive scaffolds through ES-modulated cytokine secretion from human fibroblasts.[96] In vascular TE, it was demonstrated that the proliferation of human umbilical vein endothelial cells was strongly dependent on ES intensity.[97] Also, collagen gels have been used and subjected to dynamic mechanical stimuli, and it was demonstrated that cells cultured under static stretching proliferate significantly less than that in the gels cultured under dynamic stretching.[98] Relatively to wound healing applications, mechanical and electrical[99] stimuli have been also studied. The piezoelectric scaffolds were evaluated in in vivo assays and the use of these scaffolds submitted to mechanical stimulus showed higher fibrosis level.[100] Conductive membranes have been employed in order to deliver continuous and pulsed stimuli, demonstrating the feasibility of the use of electrically activated cells in wound healing.[99]

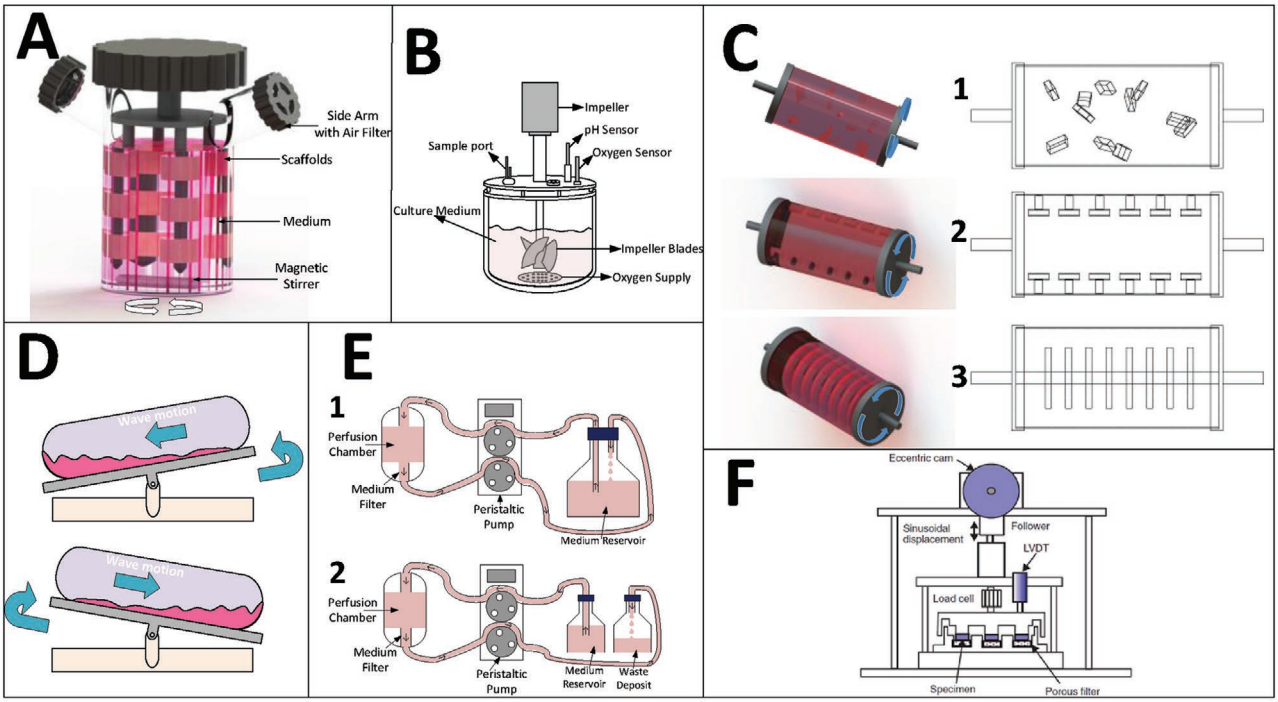

Figure 5. A) Schematic representation of a spinner flask bioreactor demonstrating how the shear stress is applied in a medium to produce convection. The cell constructs are attached in needles. Reproduced with permission.[147] Copyright 2011, Mary Ann Liebert. B) Schematic representation of the main components for cell cultivation in a stirred tank bioreactor. Reproduced with permission.[148] Copyright 2017, IntechOpen C) Wall vessel bioreactor drawing models where arrows point out the rotation direction: C1) Free-fall variation; C2) Scaffolds attached to outer vessel wall; and C2) Rotating bed bioreactor variation. Reproduced with permission.[147] Copyright 2011, Mary Ann Liebert D) Working principle of a wave bioreactor. Reproduced with permission.[149] Copyright 2008, Springer E-E1) Perfusion bioreactor setup flow chart for closed loop and E2) perfusion bioreactor setup flow chart for single pass method; F) Schematic representation of a cyclical compression bioreactor for bone marrow derived MSCs. Reproduced with permission.[150] Copyright 2004, Wiley.

Finally, mechanical stimuli have been investigated for the tendon. Different types of mechanical stimuli were provided: dynamic stretch that promoted total collagen secretion and tenogenic differentiation[101] and pulsatile strain by an electromagnet shows a preferential cell alignment at short times.[102] Both investigations show the potential and even need of the use of mechanical stimulation thought adequate scaffolds for promoting proper tendon regeneration.

4. Physically Active Bioreactors—Main Types

Bioreactors represent a useful tool for engineering physiologically functional tissues in vitro by resembling the microenvironmental niche conditions. These devices possess multiple configurations according to the tissue target type, the geometry of the scaffolds, production quantity, processing time, and operating costs (**Figure 5**).

Most of bioreactors are developed in the framework of scientific experiments according to their specific needs. From the simple bioreactor such as the basic spinner flask, to the most complex one with two or three types of simultaneous cellular stimuli by assembling together different synchronized mechanisms, these devices have been valuable for the advances in regenerative medicine. Thus, in the following section, the main types of bioreactors will be described according to their application objectives, operating methods, and main therapeutic targets (**Table 2**).

4.1. Stirred Bioreactors

Stirred bioreactors are constituted by cylinder containers where scaffolds and cells are coupled with a mechanism inside the liquid medium, which create medium motion in order to distribute evenly the nutrients, heat and shear stress stimuli among all scaffolds.

4.1.1. Spinner Flask Bioreactor

Starting by the most basic shape of bioreactor, the spinner flask is one of the most simple and most used ones, which is optimum for static condition cultures and are being mainly used for cartilage and bone tissue cultures.[14a,b] One of the main advantages of this type of bioreactor is its capacity for allowing a perfect mixed environment inside the balloon, reducing the stagnated layer of cells, thus avoiding an uneven environment. Scaffolds are suspended in needles and then placed in the middle of the flask while the environment is agitated with a magnetic stirrer, inducing the nutrient mix with the scaffold (Figure 5A).

Table 2. Representative works using bioreactors providing different physical stimuli for TE.

Stimuli	Category	Technology	Tissue type	Cell type and scaffold	Year	Refs.
Shear stress	Stirred bioreactors	Custom sample holder magnetic Teflon disk stirrer	Cartilage	Rabbit MSCs seeded into fibroin sponge	2007	[185]
		Spinner flask		Human adipose-derived stem cells seeded into fibrin gel	2012	[153]
				Trachea cartilage grafts using marrow MSCs seeded into PLGA copolymer	2010	[186]
				Bovine articular chondrocytes seeded into polyglycolic acid (PGA)	1998	[187]
				Human chondrocytes seeded into chitosan	2012	[188]
			Bone	Human bone marrow stromal cells seeded into coralline hydroxyapatite	2007	[151]
	Rotating wall – microgravity	Rotating wall vessel—free fall	Cartilage	Human articular chondrocytes seeded into a 3D porous material consisted of collagen, hydroxyapatite, and chondroitin sulfate	2012	[163]
				Bovine and human chondrocytes seeded into 2% alginate hydrogel	2006	[162]
			Bone	Rat calvarial osteoblast cells seeded into PLGA	2004	[164]
				Rat osteoblasts seeded into cytodex-3 microcarriers	2007	[189]
			Skin	Human epidermal stem cells seeded into cytodex-3 microcarriers	2011	[190]
	Perfusion	Continuous and variable flow rate within a controlled range	Cartilage	Human chondrocytes seeded into PLA	2011	[173b]
		Closed loop continuous medium flow		Human articular chondrocytes seeded into Hyaff-11 nonwoven mesh	2010	[173a]
				Human articular chondrocytes seeded into polyactive foams and Hyaff-11	2003	[191]
				Bovine articular chondrocytes seeded into 3D collagen sponges	2001	[192]
		Variable flow within a controlled range	Bone	Bone marrow-derived human MSCs seeded into fully decellularized bovine trabecular bone	2008	[172]
		Closed loop continuous medium flow	Vascular	Rat aortic smooth muscle cells seeded into decellularized rabbit aortas	2012	[193]
		Pulsatile bioreactor with endoscopic monitoring unit		Human vascular endothelial cells (ECs) and fibroblasts seeded into polyurethane scaffolds	2012	[194]
Biomechanical hydrostatic pressure	Dynamic hydrostatic	Air pressure-driven piston, custom-made stainless-steel culture chamber and mechanical manometer	Cartilage	Bovine articular chondrocytes seeded into synthetic 3D porous degrapol	2008	[195]
	Dynamic hydrostatic	Air pressure-driven piston with chamber and medium replenishing peristaltic pump		Human nasal chondrocytes and human adipose stem cells seeded into gellan gum hydrogels	2012	[196]
	Static hydrostatic			Bovine articular chondrocytes	1994	[197]
	Static and pulsed hydrostatic			Bovine articular chondrocytes seeded into agarose gels	2003	[198]

(*continued*)

Table 2. (Continued)

Stimuli	Category	Technology	Tissue type	Cell type and scaffold	Year	Refs.
Electromechanical	Compression	Stepper motor displacement, uniaxial actuator		Human chondrocytes seeded into agarose disks	2000	[199]
				Rabbit chondrocytes seeded into chitosan/gelatin	2009	[200]
				Rabbit chondrocytes seeded into genipin-crosslinked chitosan/collagen	2013	[201]
				KUM5 cells seeded into macro porous poly(ε-caprolactone)	2015	[174]
		Stepper motors displacement, biaxial actuators		Porcine chondrocytes seeded into agarose gels	2013	[177]
		Linear variable differential transformer (LVDT), uniaxial actuator	Bone	Rabbit bone-marrow MSCs seeded into agarose disks	2004	[150]
	Stretching	Vertical tube chambers with attachment anchors with translational and rotational strain		Bone marrow aspirates (human or bovine) seeded into collagen gels	2002	[202]
		Linear motor-driven uniaxial stretching device	Skin	Human foreskin expansion	2008	[203]
		Controlled 3D left-ventricular stretching through inflatable latex balloon	Muscle	Rodent neonatal cardio-myocytes seeded into decellularized rat hearts	2017	[204]
		Stepper motors pulling 4 holders for equibiaxial planar stretching		NIH 3T3 fibroblasts seeded into collagen gels	2018	[98,205]
	Electrical	Modified Cooper tissue culture disk	Bone	Osteoblastic cells (MC3T3-E1)	1997	[206]
		Arduino Uno controlling signals instrumented with digital potentiometers and amplifiers, with extra piezo-actuated micro pump for perfusion	Muscle	Cardiac cells seeded into collagen sponges	2013	[207]
		Silver electrode and platinum electrode in each end of scaffold	Nerve	Nerve stem cells seeded to polyaniline with poly (ε-caprolactone)/gelatin	2009	[208]
		Platinum mesh electrodes on culture chamber	Neural	Anionic dopant dodecylbenzenesulfonate seeded into polypyrrole	2015	[209]
	Electrostretching	Stepper motor displacing thin stainless-steel rod inserted in syringe working as a seal against contamination, stainless steel electrodes at both sample grips	Muscle	Cardiomyocytes seeded into collagen gels	2005	[181]
		Electrodes wrapped around tube inflation controlled by solenoid valves		Mouse skeletal myoblasts (C2C12) seeded into aligned electrospun polyurethane fibers	2008	[183]
		Stepper motor-driven uniaxial stretching device and electrodes of Teflon-coated silver wires		Lewis rat MSCs seeded on decellularized porcine myocardium	2013	[210]
		PDMS coated magnets grips displacement through magnetic attraction controlled by linear motor, grips with platinum wires for electrodes connections		Cardiac adipose tissue-derived progenitor cells seeded into fibrin hydrogel	2016	[92]
Multistimuli	Electro-stretching-perfusion	Stepper motor controlling grips position with electrode contacts, including single pass perfusion system		Rat cardiac progenitor cells seeded into poly-glycerol-sebacate (PGS) cardiac patches	2013	[182]
	Stretch-perfusion	Cyclic distension achieved by pulsatile medium flow	Skin	Neonatal human dermal fibroblasts (Invitrogen) seeded into fibrin gel	2015	[211]

(*continued*)

Table 2. (Continued)

Stimuli	Category	Technology	Tissue type	Cell type and scaffold	Year	Refs.
	Compression–stretching	Compression piston in one axis and grips attached to another piston in other axis	Bone	Patients hip bone marrow cells seeded into collagen type 1	2009	[212]
	Microgravity-perfusion	Perfusion chamber with biaxial rotational bioreactor for homogenous shear stress generation		Human fetal MSC seeded into tricalcium phosphate	2009	[165]

Some experiments can be found in literature using this functional basic type of bioreactor: for bone tissue enhancing osteoblastic markers within coralline hydroxyapatite scaffolds for human MSCs reaching significant higher cells count for 500 μm pore scaffolds;[151] for cartilage constructs using PLGA scaffolds for rabbit MSCs ensuing in tubular tracheal grafts formation [152] and fibrin gels as scaffolds for human adipose stem cells (ASCs) resulting in chondrogenic differentiation.[153] Spinner flasks have mainly two variation culture setups, first as in batch culture, a closed type of culture mode that disables the addition of fresh medium and waste removal, limiting production but providing a solid method to avoid contamination, whereas the second setup of continuous culture enables waste removal, but puts the system susceptible to potential contaminations.[14a] However, spinner flasks may not always be the most appropriate tool because the constant flow may induce a turbulent flow inside the capsule causing a high risk of tissue deformation and resulting in an external fibrous capsule in the tissue. Thus, they have been mainly used for small-scale production.[154]

4.1.2. Stirred Tank Bioreactor

Due to their high productivity, stirred tank bioreactors are one of the most important ones for industrial production. These systems can be used to produce red blood cells in an attempt to reduce the hospital's dependency in blood donations and thus keep a sufficient stock. Production of red blood cells is already under research with cord blood derived $CD34^+$ cells.[155] It has similarities with the spinner flask version, consisting in a cylindrical recipient with a central shaft coupled to a motor, which supports one or more stirrers. The stirrers are necessary in order to accomplish a large range of functions such as control of the proper thrust, provide the mass from stirred particles and heat transfers, to mix and homogenize the cell culture. Their working principle relies in a motor coupled to stirrer blades, which when activated mix the culture environment while being pumped to the bioreactor tank (Figure 5B). This tank is coated by a thermic mesh to avoid temperature variations in the tank. The whole system is covered by sensors in order to evaluate and monitor the culture process by controlling the variables on the tissues.[156]

Studies using stirrer-based bioreactors as cell microcarriers for MSCs have been shown to induce cell expansion and differentiation, and have been used to monitor proliferation, metabolic status and phenotype expression during suspension cultures.[157] The conventional stirrers used in fermentation are typically classified as axial or radial flow stirrers. Inside the radial flow stirrers, there are two types of turbines, one of six plane blades (Rushton disc) and other of six curved blades. The stirrers of axial flow are helixes, usually composed of three rounded blades, and a turbine with four flat blades with a 45° inclination in each. An example of use of this bioreactor, for nonmedical purposes, was for the extraction of proteins with proteolytic activity from *jacaratia mexicana* fruits reaching a cell growth of 44%, employing with Rushton turbine impellers, stirring at 300 to 400 rpm.[158] In these devices, it is possible to find stirrers with two or three turbines in the axis, reaching a higher uniformity mixture.[159]

4.2. Rotating Wall Vessel Bioreactor

The rotating wall bioreactor was initially developed by NASA (National Aeronautics and Space Administration) in order to protect the cell culture experiments from the forces to which they were subjected in the takeoffs and landings of spaceships.[160] However, later on, the device proved to be useful for TE as well, being employed to cartilage, bone, and skin tissue cultures.[14b] It consists in a cylindrical chamber where the wall is able to rotate in a constant angular speed. The cylinder wall is rotated at such speed, that it is capable of achieving balance between gravitational forces, hydrodynamic dragging force and centrifugal force, originating a micro gravitational environment, which is felt in the scaffold (Figure 5C). This balance of forces enables the scaffold to remain suspended in the cellular environment, taking full advantage of the mass transfer while reducing the shear stress.[161]

Throughout the cell culture, the tissue mass and cells number increase inside the bioreactor, raising the need to increase the rotation speed in order to equally balance the forces and assuring that the scaffold is maintained in a micro gravity environment. Experiments using this bioreactor can be found in the literature for tissues such as cartilage constructs, describing the use of alginate hydrogel as a scaffold, seeded with human cartilage progenitor cells resulting in an elastic cartilage-like tissue.[162] Scaffolds composed of collagen, hydroxyapatite, and chondroitin sulfate seeded with bovine and human articular chondrocytes have also been reported, which result is an enhanced type II collagen production.[163] Furthermore, with other types of tissue such as bone constructs, experiments can be found using poly(lactide-*co*-glycolide) as scaffolds seeded with rat calvarial osteoblast cells, where the bioreactor enables cell phenotypic expression enhancement and matrix synthesis mineralization.[164] Although the system presents no diffusion

limitations of nutrients and waste products, the cellular growth is not always uniform due to rotation, and some capsule scaffolds jump into the walls causing cellular damage to the culture (Figure 5C1). In order to overcome that, it is possible to find in the literature some derivative models such as in Figure 5C2,C3, however mineralization effects and culturing benefits due to the rotation are still limited to the scaffolds outside edges.[147] In order to overcome nonhomogeneous cellular growth, a more complex approach featuring perfusion and biaxial rotation was employed in order to generate osteogenic bone grafts with tricalcium phosphate scaffolds seeded with human fetal MSCs, resulting in a tenfold improvement regarding mass transfer in thicker grafts by comparison with static experiments.[165] Comparing static cultures and spinner flask-assisted cultures, the rotating wall vessel bioreactor presents much better results in terms of cellular proliferation and differentiation.[147]

4.3. Wave Bioreactor

The wave bioreactor is a system composed by two main parts, the stirring platform and a cell bag that receives the cellular mixture. The particularity of this bioreactor is the way its stimuli is applied by the use of waves generated by the platform to which the cell bag is attached. It is possible to control the wave motion intensity through the stirring of the motor and platform inclination.[166] These waves provide the necessary mixture and oxygen transfer to the culture, resulting in a perfect cellular growth environment, which can support more than 10 million cells per milliliter. With the angular motion of the platform, the cellular mixture keeps generating waves, stimulating the culture continuously (Figure 5D). This system provides a comparable performance to the stirred-tank bioreactors,[167] having oxygen transfer rate to Cytodex microcarrier scaffolds very well mastered, and it is possible to find in the literature studies to optimize wave rocking mechanism in terms of rocking angle, rocking rate, aeration, and mechanical design.[166] In the case of therapeutic MSCs through the combination of a single placenta, a wave bioreactor and microcarriers has been estimated to produce up to 7000 doses.[168]

One of the largest advantages of this bioreactor is the fact that there is no need of cleaning or sterilizing process of the bioreactor for each culture, due to the use of disposable cell bags, providing higher protection against contaminations and simplicity of use, also reducing the costs and time to start a production process. This only works because the culture bag is manufactured with inert biocompatible material for a single use. Another advantage of this type of systems is the capacity of production, as it offers quantities up to 500 L, being able, as well, to produce small quantities of 100 mL.[149] They are able to keep the oxygen concentrations in the small cultures, becoming comparable to the stirring tank bioreactor by its production capacities. These devices can be divided by production quantities, considering between 0.1 to 5 L for small quantities or 1 to 25 L for lab production scale. For higher quantities at an industrial scale, there are bioreactors with production capacities from 10 to 100 L and 100 to 500 L, enabling up to 5 trillion cells in a single culture.[149] However, the main disadvantage of these devices is the costs of the disposable cell bags, as the large-scale production gets too costly. Another disadvantage is the fact that each cell bag is limited to a single culture, justifying the use of this bioreactor only for high scale production in order to get the most out of each bag.

4.4. Pneumatic Bioreactor

This type of bioreactor employs gas injection for stirring and balancing the cellular culture particles. They can be used for the development of several products, like hydrogenated fat (margarine), carbonates and bicarbonates, gluconates (calcium, magnesium, sodium, and zinc), lipideous (biodiesel), biologic yeast, unicellular protein, vaccines, or fuels, among others. This device operates with several processes as well, since lipideous hydrogenation, alkalis carbonation, glucose oxidation, seaweed and cyanobacterial cultures, yeasts cultures, cell culture, or extractive fermentation, among others.[169] The pneumatic bioreactors can be divided mainly in three types: bubble column, the concentric airlift, and split airlift. These devices are mainly composed by a cylindrical tank, having in its base a sprinkler from where the bubble form gases which are further injected in the liquid.[170] Thereby, the incorporation of the gas into the liquid phase and the uniformity of the reaction environment are obtained exclusively by the injected gas, which drains upwards due its lower density, dragging with itself the liquid and promoting a random motion of the environment that further stimulates the gas–liquid mixture. In this type of devices, the rising and decreasing zone of the liquid–gas is in the same compartment, being separated in a way to drain the liquid–gas dispersion. The main difference between the bubble column and airlift-based bioreactors are the draining method. While in the bubble column bioreactor the draining process results from a random motion in the liquid phase, in the airlift version, a cyclic liquid drain is obtained.[170] A report can be found in literature where the performance of the three variations of these pneumatic bioreactor was studied, and it was concluded that the concentric tube airlift presented the higher average shear stress generation among them.[169]

4.5. Perfusion Bioreactor

Perfusion bioreactors are the ones that better simulate in vivo environment by providing a flow of medium to a cell culture, providing oxygen and nutrients through the cell-seeded scaffolds.[14c,171] These devices are mostly used in bone[172] and cartilage tissues[173] TE approaches, which take advantage of the flow generated shear stress. These devices are reported to provide better outcomes when compared with stirred flask or rotating vessel bioreactors due to higher uniformity in the mixed medium, which allows a better environment and physical stimulation on larger constructs.[13,14b] These devices are very versatile enabling several configuration types such as closed loops (Figure 5E1), where medium recirculates providing naturally produced growth factors, and single pass, which supplies always fresh medium removing any waste accumulation (Figure 5E2).[14a]

However, the medium flow must be optimized in order to avoid damaging the cell culture. High flow rates may induce high shear stress while low flow rates may induce lack of nutrients and low oxygen supply. In fact, the rate of the medium perfusion has a significant effect on tissue characteristics as it was verified in ref.[172], where bone marrow-derived human MSCs were seeded in a scaffold composed by a fully decellularized bovine trabecular bone. Low flow rates have been reported to result in higher cell viability as it has been used in[173b] (0.075 to 0.2 mL min^{-1}) with cartilage constructs composed by human chondrocytes seeded in polyglycolic acid scaffolds, but in the case of bone TE it did not accomplish a suitable distribution of nutrients, oxygen, and waste removal.[13] On the other hand, very high flow rates may hinder cell attachment as well as the formation and deposition of ECM, which then makes the perfusion system useless. Therefore, it has been suggested that the use of dynamic noncontinuous flow could be the solution for detached cells to reseed again into the scaffolds.[13] The type of scaffold used for the tissue construction will also influence the mass transfer according to thickness and pore sizes,[14c] requiring interconnected pores with 70% to 99% porosity for easier direct perfusion.[13]

4.6. Mechanical, Electromechanical, and Multistimuli Bioreactors

Several types of stimuli are felt by tissues in the human body as a result of the daily activities that result in cues such as vibration, forces, impacts, and compressions on joints, muscles and bones, as well as electrical signals that expand from brain to every part of our body. In order to resemble those conditions and thus create a biomimetic microenvironment for TE, several equipment have been designed. Single stimulus devices have been developed namely: for compression of cartilage constructs,[174] stretching of muscular based fibers[98] and electrical stimuli for human embryonic cells[175] showing better results than the ones performed in static conditions. However, the multistimuli devices, joining both mechanical and electrical stimuli are able to better mimic the conditions occurring in vivo, reaching a better biomimetic microenvironment.

4.6.1. Compression Based Stimuli

Mechanical compression bioreactors are commonly used in TE, inducing the well-known mechanotransduction phenomenon on cells. The most used one is the one providing dynamic stimulation to cells such as cartilage, offering better results by comparison with other types of stimuli for these types of tissues.[174,176] These devices are usually composed by a system of linear vertical movement with the aid of a motor and control system, which manages different amplitudes and frequencies of motor oscillations, as exemplified in Figure 5F.

The applied loads and movements frequency applied to cells in a petri box or culture plate can be defined by digital control through local control or a computer. The load is uniformly distributed to each scaffold through cylinder pins, although the height of each scaffold must be similar in order to apply the load evenly throughout the cells, to avoid an unbalanced culture.[150] However, some systems have been developed to provide dynamic shearing motions by oscillating along the *x*-axis as well, in order to spread shear stress throughout the culture wells.[177] For that, a biaxial loading bioreactor has been developed in order to stimulate cartilage tissue constructs in two directions. The authors employed an experiment of 10% of the sample thickness compression at 1 Hz on the *z*-axis, and 0.5 Hz in the *x*-axis, generating shear stress for a period of 3 h per day, reaching increased proteoglycan, collagen deposition, and the samples thickness. Although, the authors did not find a significant influence in the mechanical properties as they expected, whereas using a uniaxial compression increased both proteoglycan deposition and Young modulus.[177] Mixed type of stimuli rather than just mechanical was also developed through customized bioreactors using mechanical compression and perfusion in order to study their influence in human bone mesenchymal stromal cells contributing to equilibrium modulus enhancement and procollagen type I N-terminal propeptide synthesis.[152]

4.6.2. Stretching Based Stimuli

Stretching bioreactors use an operation principle similar to the mechanical compression bioreactors, whereas the motion also relies on a motor or magnetic displacement. This type of stimulus is being used to stimulate heart muscle,[178] smooth muscle bone tissues,[179] and tendons[180] through cyclic stretching forces. Due to better response in terms of cell proliferation and differentiation using the above-mentioned type of cellular constructs and bioreactors, the interest in applying electrical stimuli has also been a focus of study. Thus, some devices have been developed to tackle these stimulation needs in TE cultures. Some examples can be found on electromechanic bioreactors such as the one presented in ref. [181], capable of providing the tissues electrical and mechanical stimuli, employed to cardiomyocites cell culture. This device is composed by three main units: control unit, culture chamber, transform unit, and actuator of the applying forces. The culture chamber is equipped with electrodes, which provide the electrical stimuli to the tissues, with voltage amplitudes comprehended between 5 and 20 V, generated with 50 Hz frequency.[181]This unit is composed by a step-motor, which is coupled to a toothed wheel connected to another with a timing belt. This mechanism is used to connect the stainless steel rod to the motor motion, making the culture mixture inside the syringe, which is connected to the rod, to react with the electrodes in the culture chamber.[181] However, this bioreactor is limited to the quantity and culture type inserted in the syringe. In addition, for cardiac patches, other even more complex electrostretching mechanism was developed in ref. [182] able to apply cyclic strains from 0.5% to 20% at 1 to 2 Hz with square wave pulses from 6 to 8 V at 1 to 10 Hz, also including a medium recirculation system. The authors employed a validation experiment by submitting the cardiac patches through mechanical stimulation (strain 5%, frequency 1 Hz) for 24 h, evaluating the stimuli influence by comparison with static conditions.[182] In ref. [92], the authors developed an electrostretching bioreactor for cardiac adipose

tissue-derived progenitor cells stimulation, where the stretching force was generated through permanent magnets force by keeping a static end of the scaffold held by disposable polydimethylsiloxane (PDMS) supports coating neodymium magnets, where the first end was fixed and the second end was pulled by a linear movement produced by a motor. Thus, applying 50 mV cm^{-1} square waves at 1 Hz through platinum wires and 10% stretching for a period of 7 d, resulted in an improved cardiac function after myocardial infarction and increased vessel density while migrating to the murine myocardium and scar, maintaining their cardio myogenic potential as an in vivo environment.[92] In an earlier stage, complex custom setups for applying stretching and electrical stimuli on mouse skeletal myoblasts were made through tubular setups, which synchronize the electromechanical stimulation to aligned electrospun fibrous scaffolds wrapped around the silicone tubes with regulated 10 psi pressure producing up to 10.6% strain.[183] The electrical stimuli were applied through wrapped electrodes around tube endings using 10 ms square waves of 20 V, controlled by a computer running a Labview application to synchronize both types of stimuli, inducing cell elongation, focal adhesion reorganization, and stretch-activated ion channel upregulation, similar to effects previously observed during application of constant uniaxial stretch. The ES applied postmyotube formation also enhanced the myogenesis process.[183] In terms of technology development and achievement, these custom devices and multidevice assemblies already allowed important advances in the understanding of the effects of physical stimulation on cellular constructs, although still lacks more research and some quantification on ECM production according to stimuli intensity.

4.7. Magnetic Bioreactor

The magnetic stimuli bioreactor operating principle consists in applying a remote stimulus in a culture place without any mechanical attachment and reducing the risk of contamination while providing an actuation in the experiment. If the method is applied in vitro, the magnetic field can be generated outside of the tissue culture recipient, or in the case of being applied in vivo, can be generated outside the organism.[184] The magnetic signal can be supplied through a permanent magnet or electromagnet. The permanent magnets can be used in order to create an alternated magnetic field by changing their position relatively to the cells that are in culture. Thereby, the motion of the permanent magnets platform can be perpendicular to the culture chamber or longitudinal. In the electromagnets case, the position variation platform is unnecessary, as they can be controlled by current amplitude and frequency and their static presence is enough.[184] This type of system can be configured for vertical or horizontal displacement, where a culture chamber holds the scaffolds that are introduced with longitudinal space between them, and the permanent magnets are connected to a moving platform, which is connected to a computer-controlled terminal. The permanent magnets are approximated externally to the culture chamber, and each scaffold will have a magnet stimulating it. A system of this operation principle can be found in ref. [184], where the nutrients are supplied by perfusion of medium flow to the bioreactor by the entrance in the culture chamber and are directed until an exit point. Furthermore, the permanent magnets can be replaced by electromagnets, removing the need of moving parts in the system. This mechanism keeps the culture chamber fixed, making the permanent magnets platform oscillate, resulting in an alternated magnetic field applied to the culture. The usually applied oscillating frequencies vary between 0.1 and 10 Hz, even though other frequencies outside this range are possible to apply by the system. The magnet field oscillation stimulates each compression/relation cycle applied to the scaffolds, producing a motion in the nanoparticles relatively to the magnets, compressing the cells and the scaffold. With this compression, a mechanical load is simulated without direct contact. The load amplitude can be easily changed, through magnets position or changing the physical properties which compose the scaffold. The scaffold can be composed by materials that react to alternate magnetic fields and provide further stimuli as it has been observed with magnetoelectric composites based on Terfenol-D and PVDF-TrFe, where it was possible to achieve an enhancement of up to 25% in cell proliferation with preosteoblast cells.[88] However, this type of bioreactor is limited to the culture nature, meaning it only allows the use of one type of nutrients for each culture, in spite of the usual need of different cells for TE experiments in single cultures.

4.8. Microfluidic Bioreactor Approaches

In an attempt to mimic in a more reliable and reproducible way the complex in vivo cell microenvironment, also known as the cell niche, researchers have recently introduced in vitro cell culture bioreactors that combine conventional cell culture approaches with microfabrication and microfluidic technologies, also called microbioreactors that feature unique and beneficial properties that differ from conventional bioreactors. Characteristics such as miniaturization, automation and parallelization of (bio)chemical processes unleashed a rapid growth of the microfluidic technology in a wide range of fields and applications (**Figure 6**A).[213] This is sustained by their ability to integrated networks of interconnected microchannels, pumps, valves, and other components, all in a single and portable platform, which, in turn, enable the subsequent combination of a variability of analytical processes such as sampling, sample pretreatment, analytical separation, chemical reaction, analyte detection, data analysis, among others.[214] In this sense, microfluidics offer a unique opportunity to develop microfabricated bioreactors that can evaluate cells with highly complex engineered microenvironment, mediated by dynamic multifactorial cues, including molecular, structural, hydrodynamic, mechanical, electrical, among others, in a controllable, reproducible and optimizable way that cannot easily be achieved by standard bioreactors (Figure 6B).[215] In this section, it is not intended that the reader becomes an expert in microfluidic technology. For this, readers can refer to the following excellent reviews.[216] This section will provide an overview of the benefits and remaining challenges of microfluidic bioreactors compared to the conventional ones, as well as the materials and fabrication methods commonly employed. Emphasis will be given

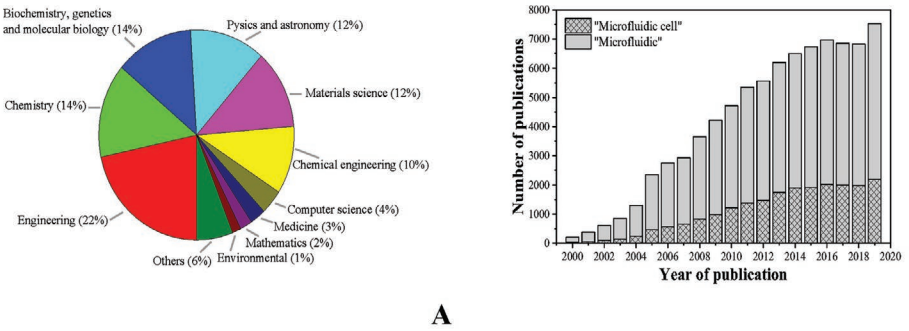

Figure 6. A) Publications related to "microfluidic," categorized by subject area, since 2000, according to Scopus database. B) Number of publications related to the design and development of microfluidic systems in general and applied to cell culture, categorized by year of publication, according to Scopus database. The graph shows relevant grow over the last 20 years.

to studies that integrate and evaluate the effects of dynamic stimuli in cell-based microfluidic platforms.

4.9. Advantages and Challenges of Microfluidic Bioreactors

Microfluidic bioreactors feature a number of important benefits comparatively to conventional macroscopic-scale bioreactors (**Table 3**). In the scope of the present section, those considered most relevant will be discussed briefly. For more details, readers can refer to the following reviews.[217] One of the most relevant is the ability of microfluidic systems to closely mimic the natural microenvironment that cells and tissues experience in living organs, through a high degree of control over cell culture conditions in various aspects, including microstructure, mechanical properties and biochemical composition.[217b] The fluidic microchannels feature dimensions at the micrometer and sub-micrometer scale comparable with those of structures and environment found in vivo such as prokaryotic and eukaryotic cells and also mass transfer length scales in tissues.[218] Under such small scale, not just study cell behavior from single cell to multicell levels can be performed, as unique fluidic phenomena occur. The Reynolds number is typically less than 100, resulting in a laminar fluid flow regime and a mass transport only by diffusion through the liquid–liquid interface, simulating the in vivo environment where all mass transport in tissues is based on the diffusion from or to blood vessels or between cells, which is a state hardly achieved with conventional macroscopic methods.[219] These conditions are ideal to study properly the biochemical and morphological responses of cells in vitro, as they can be positioned with high precision and density in a given area or volume and exposed to well-defined environment with precise spatial and temporal chemical and physical stimuli.[218] Equally relevant, microfluidic bioreactors can be designed to obtain real-time information, providing additional insights into the dynamics of specific processes in living cells.[220] A controlled cellular microenvironment with reduced contamination risks enables superior control of cellular behavior. Moreover, cells can be organized into 2D and also 3D geometries using specific integrated scaffolds based, for example, on hydrogels, allowing culture of cells in structures resembling those in tissue.[217c] Associated with the decrease of length scale is also the increase in surface area to volume ratio that enable higher diffusion rates but also faster thermal energy transfer, which should therefore be controlled to prevent abrupt temperature fluctuations. Microfluidic technology has the ability to incorporate onto a single and fully automated chip multiple steps and control systems of benchtop laboratory protocols from sample mixing, separation, capture and detection, control and readout components through reliable temperature control sensors, valves, pumps, and among others.[214b] Further, the possible assembly of an array of individual or connected controllable cell culture microchambers in a single platform enables an improved parallelization and reproducibility of assays in a high throughput manner for individual or multi cells/organs-on-a-chip[217e,221,222] models, as well as a reduction in cost and waste produced because of the small amount required, in the nL-range, pL-range, and even fL-range,

Table 3. Characteristic differences between conventional and microfluidic cell culture bioreactors.

Specification	Conventional cell culture bioreactor	Microfluidic cell culture bioreactor
Sample and reagent consumption	High	Low
Flexibility of design architecture	Fixed	High
Portability	Low	High
Capacity of single cell handling	No	Yes
Capacity of parallelization and integration of essays	Low	High
Ability of perform perfusion and chemical gradients	Low	High
Dynamic control of temperature, gases and nutrients	Limited	High
Automation of cell culture tasks	Limited	High
Real-time analysis	Limited	High
Possibilities for in situ readout of biological processes	Limited	Yes
Compatibility with established readout (robotics) equipment	Yes	Limited
Established culture protocols	Yes	Limited
Available historical experiments for comparison	Yes	Limited

of expensive samples and reagents. Furthermore, the low power consumption along with the cost effective manufacture of integrated systems using relatively low cost polymer materials provides opportunities for disposable and portable handheld devices that could potentially revolutionize the biomedical industry through unprecedented scientific research.[217g] Nevertheless, although microfluidic technology applied to cell culture has experienced noticeable growth and progress in the past 20 years, important challenges remain and must be solved in a way that these miniaturized and promising approaches become a first choice in both research and industry. This is mainly due to the unique aspects that distinguish microfluidic from macroscopic cell culture bioreactors as result of the significant changes in physical properties and large variety of possible devices designs, materials, and parameters, making direct comparison difficult if not incorrect. Thus, a proper revision of the culture protocols should be established for a greater uniformity and understanding of the obtained results. In this sense, microfluidic technology will have the potential to close an existing gap in life sciences by providing versatile, fully automated and portable analysis platforms for 2D and/or 3D cell culture with improved throughput and reproducibility, reducing hand-on-work, operating errors and need for expensive clinical samples and reagents.[217a] These highly integrated devices will find application in a wide range of biotechnological fields, ranging from basic biomedical and pharmaceutical research to point-of-care diagnostic and monitoring systems. In fact, microfluidic bioreactors containing multiple and connected organ compartments have been developed in the more ambitious organs/body/human-on-a-chip models, which may be used to asses ADME (absorption, distribution, metabolism and excretion) pharmacology.[217e,223]

4.10. Materials and Fabrication Methods of Microfluidic Bioreactors

The design and development of microfluidic platforms for cell culture have particular aspects that distinguish them from their macroscale conventional counterparts and also from microscale platforms used for other applications in biology, chemistry, or physics. The following section briefly outlines the basic components, materials and microfabrication methods used to build microfluidic bioreactors. For more extensive discussion of the mentioned topics, the readers can consult referenced reviews.[217f,219a,224] In summary, cell-based microfluidic platforms consist of two key components: cell culture platform and functional elements. One of the most important properties that any material in contact with cells should own is nontoxicity and biocompatibility. Other important features are gas permeability, surface chemistry, optical transparency, mechanical rigidity, or flexibility, rapid and cost-effective fabrication methods.[225] An overview of common materials with respective relevant properties used for the fabrication of microfluidic systems is presented in **Table 4**.

Early microfluidic platforms focused on well-established micromachining processes using silicon and glass. Silicon features a hydrophilic surface favoring adhesion of some types of cells but the lack of transparency in the visible and ultraviolet light makes it incompatible with standard microscopy methods and thus limits the development of silicon-based microfluidic culture cell platforms. Glass also has the ability to provide cell adhesion with no additional treatment as a result of its hydrophilic surface. It is nontoxic, highly transparent, available, and chemically resistant to sterilization processes.[217g] Still, both silicon and glass are brittle, gas impermeable, require expensive and time-consuming fabrication methods and bonding protocols for closing microchannels are nontrivial.[224b] Thus and despite the aforementioned advantages, which still make them popular substrates for some applications (e.g., electrophoresis), alternative materials with superior optical properties, gas permeability, biocompatibility, and low-cost fabrication methods have been successfully applied to the fabrication of promising microfluidic cell culture platforms. Within the class of polymers, PDMS, a silicon-based elastomeric material, features beneficial properties that make it one of the most employed materials for rapid prototyping of microfluidic platforms because of its low cost and easy and rapid processing with high fidelity using soft lithography methods (making used of thermoset SU-8 molds, for example).[226] Further, its biocompatibility, high gas permeability to oxygen and carbon dioxide, and excellent optical properties from the ultraviolet to the infrared (230–1100 nm) regions of the electromagnetic spectrum, have made PDMS-based microfluidic systems one of the dominant microfluidic cell culture platform.[217a] PDMS also presents high elasticity, deforming when subjected to local displacements. This property has been used not just for the easy incorporation of fluidic interconnects but also for the integration of built-in active valves and pumps[227] and to create microposts arrays that assay the mechanobiology of various cell types.[228] Despite all

Table 4. Overview of common materials and corresponding relevant properties used for the fabrication of microfluidic systems.

Material	Optical Transparency	Surface	Gas permeability	Common fabrication method
Silicon	No	Hydrophilic	No	Photolithography, chemical etching
Glass	High	Hydrophilic	No	Photolithography, wet etching
Elastomers	High	Hydrophobic	Yes	Soft lithography
Thermoset	High	Hydrophobic	No	Casting, photopolymerization
Thermoplastics	Medium to high	Hydrophobic	Yes/No	Thermomolding
Hydrogel	Low to medium	Hydrophilic	Variable	Casting, photopolymerization
Paper	Low	Amphiphilic	Variable	Printing, photolithography

these beneficial properties, PDMS features some limitations that can be challenging for cell cultures.[229] The hydrophobic surface of PDMS impairs cell adhesion, while adsorption of hydrophobic molecules can disturb the microenvironment composition and thus cell signaling dynamics.[224b] Thus, surface modification is often required to make it hydrophilic using methods such as plasma treatment, which is a technique also used to enclosed PDMS microchannels reversibly or irreversibly to PDMS itself, glass or polystyrene. Besides, the production of PDMS microfluidic platforms is not easy scalable, which make it suitable for prototyping but not trivial for commercialization. Other materials with great chemical stability and biocompatibility have been used including thermoplastics such as poly(methyl methacrylate) (PMMA),[230] polycarbonate (PC),[231] and polystyrene,[232] the material traditionally used in conventional tissue culture dishes.[217f] Fabrication methods, such as injection molding and hot embossing, are interesting option for polymer mass-production, however less exploited due to the relatively high cost of the equipment and master molds needed for replication.[233] Natural materials such as collagen,[234] fibrin,[235] agarose,[236] and other hydrogels[237] have also been incorporated into microfluidic platforms toward self-assembly of hierarchically organized cells, which provide an in vivo-like microenvironment. Nonetheless, their current fabrication techniques, such as micromolding, makes the fabrication of complex 3D networks difficult, besides needing relatively highly cross-linked hydrogels. Printing techniques, such as inkjet printing, have recently attracted attention as a way to fabricate microfluidic systems, which can allow cell-containing 3D models using suitable bio-inks, due to its automated, assembly-free 3D fabrication, rapidly reducing costs, and fast improving resolution and throughput.[238] For implantable engineered tissue constructs, emphasis has recently been given to biodegradable materials such as PLGA,[239] PGS,[240] silk fibroin,[217f] among others.[241] In addition to the aforementioned materials, inexpensive microfluidic paper-based platforms have been fabricated and tested for 3D cell culture.[242] For the foreseeable future, although PDMS will continue to provide an affordable rapid prototyping option for most researchers, the shift to new materials and techniques is primordial to create new approaches that meet all the necessary requirements for effective biomimetic microfluidic bioenvironmental platforms. Concerning the second main components of cell-microfluidic platforms, a wide range of key functional components, including micromixers, microvalves, micropumps, sensing, control, and acting elements, can be used to create effective, complex, and powerful integrated microfluidic networks.[243] Micromixers are used to overcome the low and inefficient mixing by diffusion that occur in microscale channels, in order to assure, for example, proper enzyme activity, temperature homogeneity, oxygen transfer, among others.[244] For that, passive or active micromixers can be used, where passive mixers rely on patterned geometries and/or controlled flow energy,[245] while active mixers consist on integrated magnetic, thermal, electrokinetic or piezoelectric elements, among others.[246] Although highly efficient, in these last cases, where external active forces are used, special attention should be taken to avoid cell damage. Microvalves and micropumps, in turn, are used to increase functionality by allowing a precise control of flows and fluids interactions, which can be relevant, for example, to provide a continuous and control supply of growth media in microfluidic bioreactors.[247] Sensing and control elements are key components to manufacture effective microfluidic bioreactors with the ability to monitor and/or control in real time relevant process parameters such as temperature, optical density, dissolved oxygen, and flow rate.[218] Regarding optical sensors, a wide class of detection methods can be found in the literature, which depends on the optical properties to be measured. Absorption, fluorescence, chemiluminescence, and surface plasmon resonance (SPR) are some example of techniques commonly used in microbioreactors. Others sensors include mechanical transducers, such as quartz crystal microbalances (QCM) and electrical/electrochemical sensors based on microelectrodes, amperometry, conductometry or voltammetry, among others. The main characteristics of the most used detection techniques, including working principal, advantages and limitations can be found in ref. [248]. Last but not least, functional elements, including mechanical, electrical, magnetic and/or piezoelectric acting systems, can be integrated in microfluidic bioreactors not just to directly manipulate fluids and specific compounds, including cells, as previously exposed, but also to create dynamic stimuli that allow to mimic more properly in vivo microenvironment and study their effect on cells behavior. Specific examples on the application of these stimuli in cell-based microfluidic platforms will be presented and discussed in the following section.

4.11. Applications of Microfluidic Bioreactors

The numerous benefits of microfluidic bioreactors results in their application in various fields, such as (bio)chemical analysis,[249] pharmaceutical discovery,[250] environmental monitoring,[251] DNA,[252] and proteins[253] separation and analysis. Further, they have been used to study various aspects of cell biology ranging from adhesion, spreading, proliferation and differentiation, toxicity monitoring, cell counting and sorting, signaling mechanisms, among others.[214a,217g,254] Such a wide range of possibilities can cover all the necessary requirements for a cell biology laboratory, where in vitro study of single cells, populations of cells, tissues and even whole organs is conceivable such as vasculature-on-a-chip,[255] skin-on-a-chip,[256] brain-on-a-chip,[257] bone-on-a-chip,[258] muscle-on-a-chip,[259] heart-on-a-chip,[260] lung-on-a-chip,[261] liver-on-a-chip,[262] gut-on-a-chip,[263] kidney-on-a-chip,[264] multiorgans-on-a-chip,[265] or tumor-on-a-chip.[266] In this sense, the application of microfluidic bioreactors for cells studies is growing and expanding rapidly with continuous emergence of new designs and new microenvironments using different materials, processing techniques, and adding functional elements. It is well known that cell manipulation and sorting are essential steps in cell biology research and in many diagnostics and therapeutic methods and have been accomplished in microfluidic systems using mostly biochemical labels.[254,267] Further, there have been also interest in taking advantages of other physicochemical properties of cells such as size, electrical polarity, and hydrodynamic properties. An early application of cell-based microfluidics included combining 2D dielectrophoretic forces

with field-flow-fractionation in a microfluidic device for separating cells according to their dielectric properties.[268] Another example allows continuous-flow size-based cell sorting using a microfluidic syringe filter by incorporating a series of ridges having the slanted geometry that locally produces rotational flow patterns and induces hydrophoretic particle ordering.[269] More recently, cell manipulation has been also carried out by means of external forces, including mechanical,[270] electrical,[271] magnetic,[272] and piezoelectric,[273] by integrating specific functional elements in cell-based microfluidic bioreactors. Besides this application, these acting elements can be used to generate specific stimuli not just to better mimic some cellular microenvironment, such as blood flow, vibration, forces or electrical signals, already referred under the conventional macroscale bioreactors, but also to study more properly their effect in specific cellular behaviors. Thus, this section will focused on some microfluidic bioreactors that study the effect of dynamic stimuli as indicative examples of so many others applications that can be found in a number of excellent papers, already mentioned through this review.[221,233a,242,274]

A biomimetic microsystem incorporating two side microchambers that apply mechanical strain on a center microchannel was fabricated to simulate the functional alveolar-capillary interface on a human lung, which are subjected to cyclic inflation/deflation movement during the respiration process (**Figure 7**A).[275] The application of vacuum to the side chambers generates the deflection of thin walls between the side chambers and center microchannels, which, in turn, caused the stretching of a PDMS membrane located in middle of the center microchannel. The results revealed that cyclic mechanical strain not just accentuated toxic and inflammatory responses of the lung to silica nanoparticles but also enhanced the uptake of nanoparticles into epithelial and endothelial cells and stimulates their transport into the underlying microvascular channel. An in vitro living cell-based model of the intestine was also developed to mimic relevant physiological cues, including cyclic mechanical strain, such as the one originated from peristalsis of the digestive tract, fluid flow and coexistence of microbial flora (Figure 7B).[276] Simulating the low level of fluid flow and shear stress experienced in the living intestine

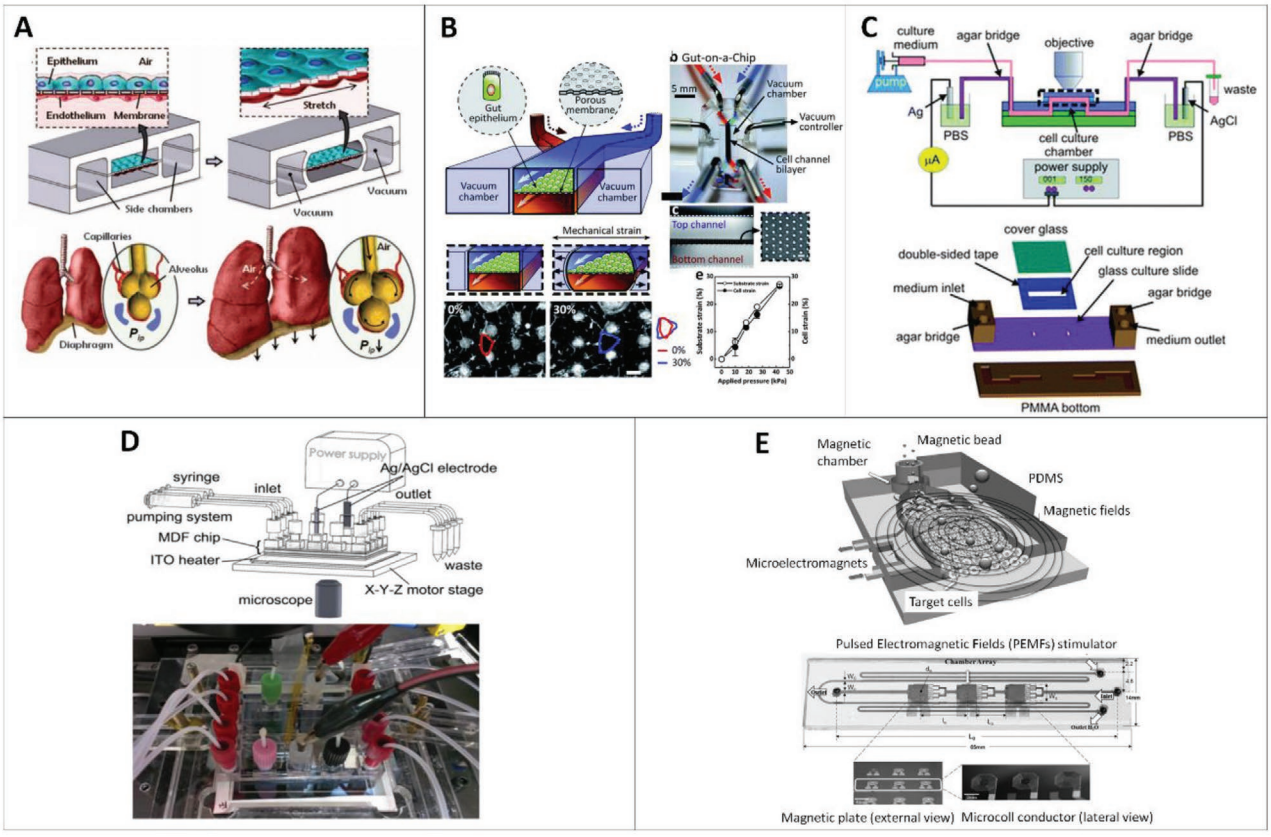

Figure 7. Examples of microfluidic bioreactor approaches that apply and study the effect of dynamic stimuli on cell behavior. A) Biologically inspired design of a human breathing lung-on-a-chip microdevice that recreates physiological breathing movements by applying vacuum to the side chambers and causing mechanical stretching of the PDMS membrane forming the alveolar-capillary barrier. Reproduced with permission.[275] Copyright 2010, American Association for the Advancement of Science. B) Biomimetic gut-on-a-chip microdevice that mimic relevant physiological cues of living intestine, including cyclic mechanical strain, fluid flow and coexistence of microbial flora. Reproduced with permission.[276] Copyright 2012, Royal Society of Chemistry. C) PMMA-based MDF platform to study the electrotaxis of lung cancer cells under chemical and electrical fields. Reproduced with permission.[277] Copyright 2011, Royal Society of Chemistry. D) MEC platform with structured-illumination nanoprofilometry to investigate the variations of filopodia of lung cancer cells under external direct-current electric field stimulations. Reproduced with permission.[278] Copyright 2014, AIP Publishing. E) PDMS-based microfluidic magnetic bead impact generator for physical stimulation of osteoblast cell. Reproduced with permission.[279] Copyright 2010, Wiley.

accelerate intestinal epithelial cell differentiation, formation of 3D intestinal villi-like structure and increased intestinal barrier function. This gut-on-a-chip demonstrates the ability to simulate multiple dynamic physical and functional features of human intestine that are critical for its function within a controlled microfluidic environment and, thus, has the potential to be a great value for drug testing and development of unique intestinal disease model. Other recent study reported on a fully automated microfluidic platform for delivering robust and precise mechanical stimuli by using pneumatically actuated structures to (worms) *Caenorhabditis elegans* (*C. elegans*), which is an important model system for the elucidation of genetic and cellular mechanisms in sensory behavior and potentially relevant to human diseases.[280] The obtained results demonstrate the potential of this system to accelerate the discovery of genes and molecules involved in mechanosensation and multimodal sensory behavior, as well as the discovery of therapeutics for related diseases. Other recent advances on enabling technologies to reproduce and study dynamic mechanical microenvironment in cell-based microfluidic platforms can be found in ref. [281]. Electric field was also used as powerful stimulus to control the movement of *C. elegans* in a microfluidic environment.[282] The exposure of worms to electric field induces their movement toward the cathode that is directional, fully penetrant and highly sensitive, allowing to precisely control and orient worms in an efficient and automated manner. This phenomenon is termed electrotaxis and referred to the movement of cells with a directional preference toward the cathode or anode under direct-current electric fields. Numerous studies have demonstrated that cancer cells undergo reorientation and migration directionally under physiological electric field, which, in turn, has potential implications in metastasis.[283] Thus, various studies were conducted in order to study the electrotaxis of lung cancer cells under the concurrent effect of chemicals and electrical fields by means of a multichannel-dual-electric-field (MDF) microfluidic cell culture chip (Figure 7C).[278,284] Besides the conclusion on cell behavior under these stimuli, the system demonstrates great potential for further investigation on the complex biological mechanisms of electrotaxis in an attempt to reveal the regulation of cancer cells movement under ES. A microfluidic electrical-field cell-culture (MEC) platform with structured-illumination nanoprofilometry was developed to investigate the variations of filopodia (needle-like protrusions from the edges of cells commonly observed on cancer cells with high mobility) of lung cancer cells under external direct-current electric field stimulations that simulate the endogenous electric field environment (Figure 7D).[277] This study demonstrated that cancer-cell filopodia respond to variations in electrical field in the microenvironment, and thus, could play a relevant role in cancer metastasis. Other interesting microfluidic platform was developed in order to quickly apply versatile ES signals to cells suspended in microfluidic channels and measure extracellular field potential simultaneously.[285] The system was able not just to noninvasively distinguish electrically excitable cells, such as cardiomyocytes cells, from electrically nonexcitable cells, such as human umbilical vein endothelial cells, but also to detect viable cells in cardiac tissue. The results demonstrate the potential of this tool to optimize the electric stimulation conditions to facilitate the functional engineered cardiac tissue development. Microfluidic dynamic microenvironments platforms combining mechanical and electrical stimuli have also been reported on literature.[286] Regarding the use of magnetic and piezoelectric stimuli in cell-based microfluidic bioreactors, few studies were found in the literature, being these actuators mostly used for cells manipulation and detection.[272–273] A microfluidic bioreactor was designed and fabricated to physically stimulate osteoblast cells using magnetic microbeads impact generated by pulsed electromagnetic field from an integrated microelectromagnet (Figure 7E).[279] The growth rate of cells showed significantly accelerate when stimulated with a proper defined beads size and pulse frequency, with almost all the cell viable after stimulation, demonstrating the suitability of the platform for use in new physical stimulus modalities. Other study investigated various aspects of cell behavior including migration, proliferation and morphological changes in a microfluidic platform composed by highly flexible magnetic composite polymer-based bidirectional actuators (M-CP) with micropatterned nonmagnetic PDMS.[287] The system was successfully tested to stimulate endothelial cells grown in the microfluidic platform with both fluid flow and mechanical stretch/compression using the new M-CP actuators. A microfluidic cell lysis platform was also designed and fabricated using a magnetically actuated micromixer to disrupt cells, a hydrophobic valve to manipulate the cell lysate, and a packed porous polymerized monolith chamber for filtering debris from the cell lysate.[288] Actuating the magnetic micromixer with an external magnetic stirrer at a frequency of 1.96 Hz generates a final cell lysis efficiency greater than 90% using recombinant *Escherichia coli* cells as model. A more recent study combined magnetic stirrer with a bead beating features for cell wall disruption of hard to lyse microorganisms in a microfluidic platform.[289] The systems feature the ability to work in continuous flow performing lysis at flow rates ranging from 30 to 180 μL min^{-1}, being the lysis efficiency higher as lower the flow rate. An optimum yielding of 43% lysis efficiency relative to off chip bead beating was obtained, using *Staphylococcus epidermidis* as target cells, with a lysis flow rate of 60 μL min^{-1}, conditions that allow a good compromise between time consumption and efficiency. Regarding the use of piezoelectric phenomenon, an acoustically driven system for tunable and deliberate stimulation and manipulation of cell growth in a microfluidic platform was developed and tested successfully for the active positioning of neurons and consequent guiding of neurite outgrowth, which allow overcoming the limitations of static approaches.[290] A microfluidic perfusion bioreactor integrating ultrasound standing wave traps formed by a piezoelectric lead zirconate titanate (PZT) transducer also demonstrates to promote the generation of 3D agglomerates of human articular chondrocytes, and enhance cartilage formation by cells of the agglomerates via improved mechanical stimulation and mass transfer rates.[291] Histological analysis and assessment of micromechanical properties confirmed that the neaocartilage grafts were analogous to native hyaline cartilage. Another acoustofluidic bioreactor platform was developed to allow the application of acoustic forces to mechanically stimulate aggregates of human articular chondrocytes in long-term levitated culture (Figure 5F).[292] The oscillatory fluid shear stresses generated by the PZT-based acoustic stimulus, combined with suitable biochemical cues have the ability to engineer human cartilage

constructs with structural and mechanical properties analogous to those of native human cartilage.

5. Final Remarks and Future Perspectives on Tissue Engineering Devices

An adequate supply of nutrients to cells in culture is one of the most important parameters to be addressed in TE. When implanted in a patient, an engineered material or tissue do not possess an established vascular network and the transport of nutrients are critical during the initial period after implantation. This review thus shows the potential of using bioreactors applying physical stimuli to improve the outcome of in vitro fabricated scaffolds for TE applications in order to recreate proper cell microenvironment during cell proliferation and differentiation.

Bioreactors are devices which, in order to get the most suitable results should be designed/adapted for each specific TE experiment in order to promote cellular high proliferation rates and provide each process step, such as seeding or harvesting, while avoiding contamination. However, in order to reach acceptable developing time and allowing a suitable interpretation of the obtained results, an in-between compromise has to be achieved in order to reach a viable solution with enough degree of freedom according to each specific set of experiments.[293] An important aspect relies in control and system variables awareness through closed loop control where the system reads its own actuation and acts according to the measured error correcting itself, in order to provide the most accurate possible actuation on each culture.[293b] The errors may have several sources, since internal or external vibrations, closure of bioreactor's door or incubator or simple system components aging or degraded from heat, which may influence tissue progression through nonintended mechanobiological signals being applied to the cells introducing bias in the analysis.[293b] These are the drawbacks that need to be taken into consideration in future bioreactors development for TE purposes.

Despite all that, designing these bioreactors have led to promising advances not only for delivering essential nutrients to the cells but also for acting as an active platform that provides environmental cues in a biomimetic approach to drive tissue development. The possibility of growing a tissue in vitro using bioreactors has been considered a milestone in regenerative medicine since the scaffolds do not function only as a support for living cells or as a vehicle for growth factors but also assist in the process of tissue formation and/or regeneration.

Nevertheless, the development of biomimetic scaffolds and bioreactors is still very challenging and requires specific improvements to permit the clinical translation of these technologies to regenerative medicine. Furthermore, this kind of technology can be use in order to support the production or manufacture of cell-based products in a clinically and commercially viable way, helping for example ex vivo cell therapies, such as patient-specific approaches.[294]

In this way, several requirements must be fulfilled for the design of a device for TE, since the construction materials, which must be biocompatible and chemically resistant, but also able to withstand the sterilization process required before each culture, such as UV light radiation, autoclave and specific disinfection processes. Thus, each tissue-specific culture conditions requirements must be sufficed regarding also, experiment process, oxygenation, nutrient supply, and stimuli amplitude range.[293a] The types of intended stimuli to be applied to the culture plates/chambers must be carefully studied in order to accomplish the most suitable range of stimuli according to the type of tissue targeted and respective geometry directly related to scaffold size, porosity, topography and stiffness.[14a] In addition, scaffolds can be used as electromechanical actuators as well, when composed by smart electroactive materials,[88] working symbiotically with a bioreactor for single or multistimulation operation on the tissue culture. The same type of stimuli can be applied through more than one method, as an example, stretching stimuli type of bioreactors, can use motor driven clamps, pressurized fluids or gas pumps to apply uniform stretch on scaffolds. However, pumps can be used outside incubators to provide the mechanical stress instead of motors, gaining space inside the incubator and avoiding inside generated heat.[295] These pump-based setups can be used to produce tissues such as heart, lungs, bladders, or vascular vessels, although, among stretching, some stretching movements require motor driven clamps in order to produce rotation at one end while stretching in the other, which is useful for engineered tendons and ligaments, while applying direct contact to apply electrical stimuli.[295] The quality of force/stress measurement is key in order to precisely quantity the correct relation between stimuli and cellular growth in the scaffolds.[293b] A very important aspect still relies in sensors used for culture monitoring and precise stimuli control, in order to keep track of the closest possible quantification of the amount of stimuli applied to the cellular constructs to get a correct correlation between stimuli and cell proliferation/differentiation. However, in this type of experiments many variables exist, which may make replications harder to accomplish under precise similar conditions (including oxygenation, mechanical stimuli, nutrient distribution, or medium pH related to waste quantity, among others).[14c] Nevertheless, the most suitable conditions for each type of therapeutic grafts require further research to understand and define, through the use of increasingly more complex and multi-stimuli bioreactor devices, in order to overcome these limitations. The study of anisotropic mechanical properties through real-time monitoring systems has been suggested,[295] although the costs associated with digital image correlation and high speed cameras does not really tend for affordable medical implants production, it still may be an interesting approach for research and cellular behavior analysis.

While perfusion bioreactors are the ones providing the closest environment to in vivo conditions, the bioreactors lack the flow of medium that allows the oxygen and nutrients reach the cells within the scaffold. In Table 1, related to the previously described bioreactor variations, it is possible to get an overview of what type of technologies are being employed in research and with which type of TE grafts are they associated with according to the category stimuli they provide.

Culturing cells in microfluidic devices holds great promise in terms of technology for bioreactors development but it has been poorly reported. It requires an understanding of

certain fundamental principles that span multiple disciplines, including biology, biochemistry, physics, and engineering thus it needs a multidisciplinary actuation. While the past 5 years have seen significant strides in each of these key areas, the overall impact of microfluidics on modern biology continues to be marginal, and much of the potential of microfluidics that was promised continues to be largely unfulfilled. This is apparent from the lack of widespread acceptance within the biology community to adopt microfluidic methodologies into their laboratories. To advance the field toward more mainstream acceptance, it is beneficial to discuss the current state of the art, evaluate the recent progress within these main areas of development, and identify remaining challenges that need to be addressed in order for microfluidics to become truly useful to biologists.

The challenges related with the development of the next generation of bioreactors and scaffolds should thus contemplate the following considerations: i) taking into consideration the vascularization of tissues in vitro should be a priority in these experiments, which would further allow them to be properly prepared for vascularization in vivo, upon implantation; 2) it is important to include an inflammatory milieu together with the growing tissue for optimal tissue development, since inflammation is an essential component of the normal mammalian host tissue response and need to be present for a biomimetic approach; 3) and last but not least, a constant monitoring of the bioreactor environment and tissue development using advanced imaging and sensing modalities is important to monitor cell fate and tissue development in the 3D complex environment. Real-time and nondestructive assessment of tissue and organ regeneration will be essential to the eventual automation of bioreactor control. Imaging and sensing readouts may be used in a feedback loop to signal inputs of environment cues (e.g., mechanical actuation, oxygenation) or delivery of biological factors.

This recent engineering field is not only providing interesting results, but has also demonstrated to have much room for improvement by assembling different types of stimuli mechanisms together in order to keep improving these biomimetic apparatuses over the next years, allowing new and essential insights on tissue regeneration and allowing more suitable TE strategies.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

bioreactors, cell microenvironment, microfluidic, physical stimuli, smart materials

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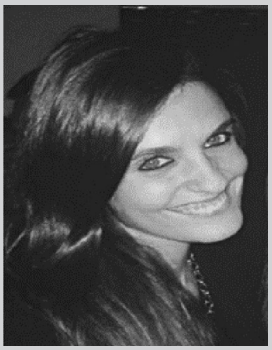

Sylvie Ribeiro is a researcher at the Physics Center of the University of Minho, Portugal. She graduated in Biological Engineering at University of Minho, being her master thesis done in Institut National des Sciences Appliquées (INSA) in Toulouse and she obtained her Ph.D. degree in Engineering of Materials at the same institute in Portugal. Her work is focused in the area of interface between biomaterials and muscle cell types for tissue engineering applications with the use of smart materials and bioreactors to create suitable cell microenvironments.

S. Lanceros-Mendez is Ikerbasque Professor at the BCMaterials, Basque Center for Materials, Applications and Nanostructures, Leioa, Spain, where he is the scientific director. He is associate professor at the Physics Department of the University of Minho, Portugal (on leave). He graduated in Physics at the University of the Basque Country, Leioa, Spain and obtained his Ph.D. degree at the Institute of Physics of the Julius-Maximilians-Universität Würzburg, Germany. His work is focused in the area of smart and multifunctional materials for sensors and actuators, energy and biomedical applications.