Chapter 15

Tailoring Bioengineered Scaffolds for Regenerative Medicine

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Additional information is available at the end of the chapter

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Abstract

The vision to unravel and develop biological healing mechanisms based on evolving molecular and cellular technologies has led to a worldwide scientific endeavor to establish regenerative medicine. This is a multidisciplinary field that involves basic and preclinical research and development on the repair, replacement, and regrowth or regeneration of cells, tissues, or organs in both diseases (congenital or acquired) and traumas. A total of over 63,000 patients were officially placed on organs' waiting lists on 31 December 2013 in the European Union (European Commission, 2014). Tissue engineering and regenerative medicine have emerged as promising fields to achieve proper solutions for these concerns. However, we are far from having patient-specific tissue engineering scaffolds that mimic the native tissue regarding both structure and function. The proposed chapter is a qualitative review over the biomaterials, processes, and scaffold designs for tailored bioprinting. Relevant literature on bioengineered scaffolds for regenerative medicine will be updated. It is well known that mechanical properties play significant effects on biologic behavior which highlight the importance of an extensively discussion on tailoring biomechanical properties for bioengineered scaffolds. The following topics will be discussed: scaffold design, biomaterials and scaffolds bioactivity, biofabrication processes, scaffolds biodegradability, and cell viability. Moreover, new insights will be pointed out.

Keywords: tailored scaffold, biomaterials, bioprinting, biomechanics, regenerative medicine

1. Introduction

In a society that is in constant development, the discovery of "new" scientific and technological knowledge must (i) progress at an incredibly fast pace, (ii) target a wide audience, and (iii) have a practical impact in the society. The health sciences are naturally a priority area of

research, mostly because of the impact they have on the augment human life expectancy, by developing advanced and patient-specific therapies.

Only the complexity of human tissues could justify that in the 1980s tissue engineering emerged as a scientific field with an enormous potential. Targeting to regenerate the bone, cartilage, skin, or other tissues and organs, bridging the anatomy with its physiology/function is a paramount challenge to be solved. Several efforts have been made, by research groups spread worldwide, to tailor bioengineering scaffolds (sometimes denominated by tissue constructs) that could mimic native tissues. However, the achievement of three-dimensional (3D) complex organ structures is far from being tangible. Due to its nature, tissue engineering gathers scientists, engineers, and physicians in multidisciplinary teams using a variety of methods to construct biological substitutes [1]. Indeed, significant efforts are being developed worldwide in the fields of tissue engineering and regenerative medicine, but full tissue or organ regeneration remains a paramount challenge. Therefore, these multidisciplinary scientific fields apply a wide variety of methodologies, where multidisciplinary research teams can provide suitable inputs for its development [2].

One of the major goals is to produce biological substitutes to restore, maintain, or improve tissue function, using biocompatible and biodegradable support structures, i.e., scaffolds, in conjunction with human cells (**Figure 1**) [3]. Gathering tissue engineering and regenerative medicine, researchers have been interested on developing alternative approaches for restoring functionality. To do so, one of the most promising methodologies involves the use of additive manufacturing (AM) processes. AM technologies allow the production of complex 3D structures concerning mainly a high level of control, predefined geometry, size, and interconnected pores in a reproducible way. This optimized controlled organization enhances the vascularization and, thus, transports oxygen and nutrients throughout the whole structure,

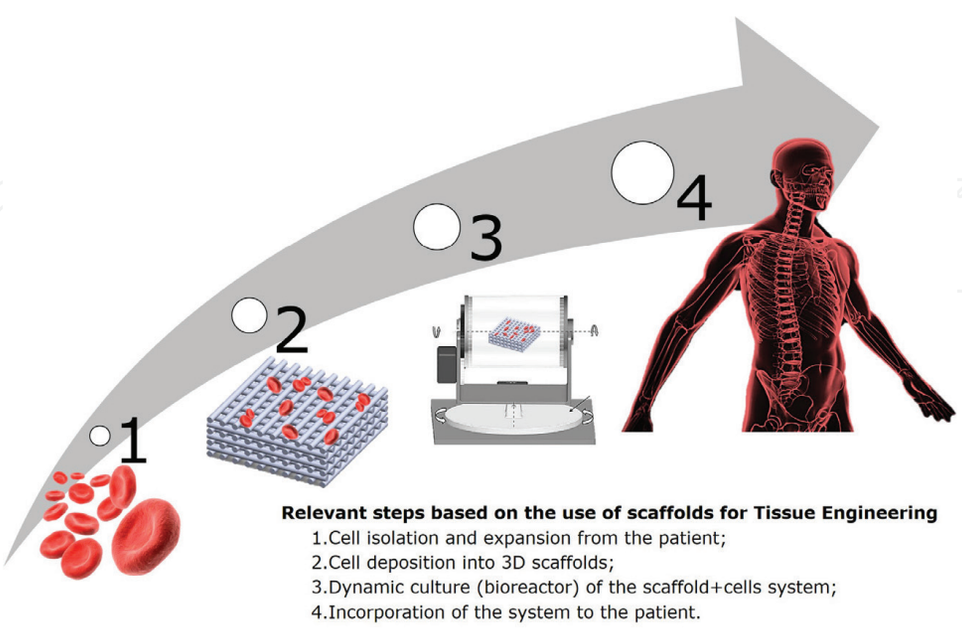

Figure 1. Relevant steps based on the use of scaffolds for tissue engineering.

providing an adequate biomechanical environment for tissue regeneration [4]. However, adapting the adequate technology with enhanced biomaterials, in order to obtain customized implants that mimic the native tissue, is nowadays a challenge with a huge potential.

This chapter intends to provide a synopses in patient-specific engineering scaffolds. A revision of the scaffold design, biomaterials, and advanced manufacturing processes will help to establish new research paradigms on tailoring bioengineered scaffolds for regenerative medicine. Recent advances will be highlighted to stimulate the readers for future insights and possibilities.

2. Scaffold design

Scaffold modeling plays a key role in tissue engineering and regenerative medicine. A well-designed 3D scaffold is a fundamental tool to guide tissue formation both in vitro and in vivo. Properties such as high surface-area-to-volume ratio, porosity, pore size, pore design, pore interconnectivity, permeability, and degradation should be taken into account when designing scaffold for different and tailored applications. These will allow a desirable biological network for cell migration, nutrient transportation, and the mechanical stiffness, and strength can be therefore obtained [5, 6]. Growth factors (GFs) and drug release (DR) should also be considered to achieve an optimized tissue growth as scaffold degraded. Moreover, some authors have shown the benefits for tissue generation of using curvature and concave surfaces compared to convex and planar ones [7].

To address and fulfill aforementioned requirements, two scaffold design approaches can be used according to the flowchart presented in **Figure 2**. The first one is based on the native tissue, whereas the second one is based on the unit digital cell model, both addressing tailored scaffold geometry. The geometry obtained can then be used on computer-aided engineering studies to optimize the performance of the tailored bioengineering scaffold. Finally, a physical optimized scaffold can be produced using 3D printing or AM technology before in vitro and/or in vivo implantation of the scaffold. Accordingly, several research works have been developed concerning tailored scaffold geometry and its fabrication. In these studies, physical scaffolds have been used directly for in vitro and/or in vivo studies. Nevertheless, the link between computer modeling and computer-aided engineering to tailor bioengineering scaffold remains a paramount challenge. When solved, it can significantly reduce animal experimental studies.

In the computer modeling based on native tissue, different noninvasive 3D scanning techniques can be considered to obtain the 3D anatomical geometric model. The most used are computed tomography (CT), μCT (micro-CT), and nCT (nano-CT), which considered different scale levels [8–10], as well as magnetic resonance image (MRI) and 3D optical techniques. All these techniques used different physical principles to obtain a series of two-dimensional (2D) images or a 3D point cloud of the sample of the native tissue studied. CT requires the exposition of the sample of the native tissue to ionizing radiation, whereas MRI uses a magnetic field and pulses of radio wave energy (avoiding radiation) both obtaining a series of 2D images. In

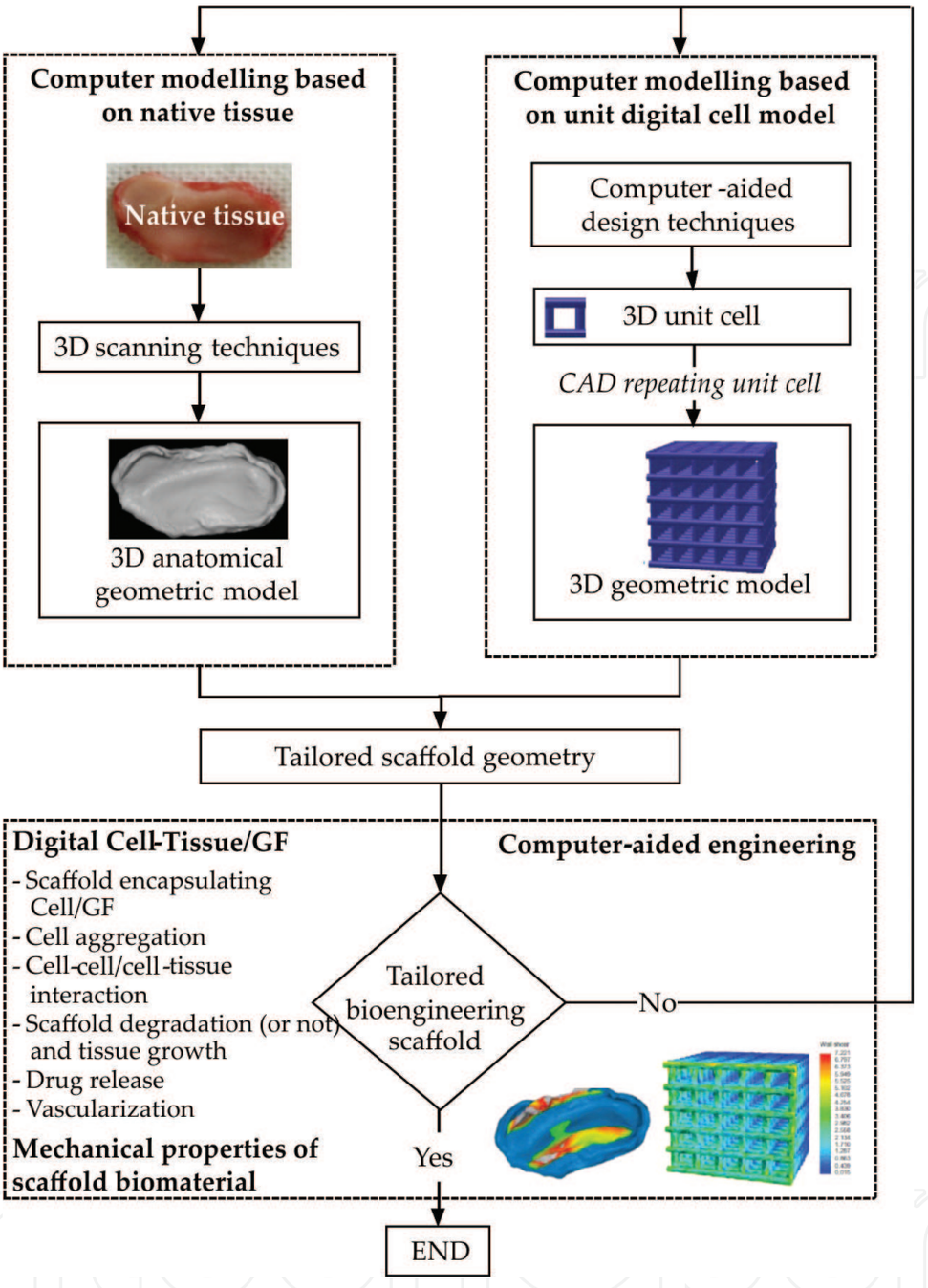

Figure 2. Computer modeling and simulation to tailor bioengineering scaffolds.

CT, these images are displayed by density, while in MRI they are compiled and segmented by its signal intensity. Additionally, both techniques can be differentiated by its resolution. The high resolution of CT allows the characterization of the micro-architecture and the mechanical properties of the tissue scaffolds [11]; however, this technique has a drawback regarding soft tissues of similar density. It is more efficient in differentiating hard tissues with sharply defined density changes, such as the interface between bone and soft tissues. To overcome this problem, contrast agents can be added [6]. Although the resolution of MRI is inferior to CT scans, with the advance of technology, it is improving, allowing the 3D representation of

internal structures, such as the central nervous system, heart, and kidneys of a rat [9]. The 2D individual images obtained using the previous techniques described are then assembled and realigned, and therefore a 3D geometric anatomical model distinguishing different types of tissue is obtained.

Microscopy optical technique is also used to obtain the 3D anatomical geometric model of the native tissue. However, it can only differentiate every type of tissue down to the level of the individual cell at the cost of a huge computational effort. Other 3D optical techniques, such as 3D structured light, cannot differentiate the types of tissues presented and only allow the generation of the outer 3D geometric model of the native sample. In the case of the native tissue sample (temporomandibular joint disc (TMJ disc)) presented in the flowchart of **Figure 2**, a 3D point cloud was obtained using a white light 3D scanning system (Steinbichler—COMET 5®), and then an appropriate software was applied to replicate the 3D geometric anatomical model of the TMJ disc [12].

Hybrid modalities can also be used to construct the 3D model of the same specimen in order to take the advantage of each technique to differentiate the different types of tissues [9].

The second approach uses computer-aided design (CAD) techniques to create a 3D unit cell which is used as pattern. Then, a desired number of patterns are automatically generated and combined until a complete 3D geometric model of the scaffold is obtained with controlled architecture (**Figure 2**). Following this approach, the main scientific achievements reported are based on permanent or temporary scaffolds.

Permanent tailored engineering scaffolds have been designed mainly for bone repair of large segmental defects caused by fracture, tumor, or infection. In 2013, Wieding et al. [13] reported a numerical study which is used to determine the suitability of open porous of titanium scaffolds to act as bone scaffolds under physiological loading conditions. Uniaxial compression structural modulus of the titanium scaffolds was tailored ranging from 3.5 to 19.1 GPa as a function of the scaffold porosity from 64 to 80%. Results revealed that minimizing the amount of material of the inner core had a smaller influence than increasing the porosity when the scaffolds were under biomechanical loading. It was also noted that the scaffold design could act similarly to the intact bone. In order to tailor the mechanical properties of cellular structured scaffolds, [14] designed metal scaffolds with high porosity (62–92%) to tailor both compressive strength (4.0–113.0 MPa) and elastic modulus (0.2–6.3 GPa), respectively, were comparable to trabecular and cortical bone. Porous titanium scaffolds were also investigated by van der Stok et al. [15, 16] for grafting large bone defects. Mechanical properties were tailored, whereas high porosity of the scaffold allowed the incorporation of colloidal gelatin gels for time- and dose-controlled delivery of dual growth factors (bone morphogenetic protein-2 (BMP-2) and/or fibroblast growth factor-2 (FGF-2)), promoting a quasi-full bone regeneration. The scaffold was designed based on a decahedron pattern and composed by 120-μm-thick titanium struts with porous size ranging from 240 to 730 μm. Porous size, porosity, porous volume, compression strength, and Young's modulus were 490 μm, 88%, 55 mm^3, 14 MPa, and 0.4 GPa, respectively, allowing to achieve an optimized bone volume regeneration (~50 mm^3) for a composite scaffold with BMP-2/FGF-2. In 2012, Van Bael et al. [17] developed six distinct geometries of Ti6Al4V scaffolds in three different pore shapes (triangular, hexagonal, and rectangular) and two different

pore sizes (500 and 1000 μm) aiming to understand the effect of pore geometry of Ti6Al4V bone scaffolds on the in vitro biological behavior of human periosteum-derived cells. The main result showed that a functional Ti6Al4V-graded scaffold, with specific morphological and mechanical properties, will contribute to enhance cell seeding and at the same time can maintain nutrient transport throughout the whole scaffold during in vitro culturing by avoiding pore occlusion.

Temporary (or biodegradable) tailored engineering scaffolds have been designed as a tissue engineering approach that uses degradable porous biomaterial incorporating biological cells and/or molecules to regenerate tissues such as the bone, cartilage, skeletal muscle, nerve, and blood vessels. Scaffold design must be able to create hierarchical porous structures to fulfill all mechanical and biological requirements. In 2005, Hollister [18] introduced the concept of hierarchical scaffold design as geometric features at scales from the nanometer to millimeter level that will determine how well the scaffold meets conflicting mechanical function and mass transport needs. In 2011, Khoda et al. [19] developed a functionally gradient variational porosity architecture (hierarchical design) for hollowed scaffolds. In 2014, Giannitelli et al. [20] reviewed tailored scaffold architecture with microstructural features. Authors highlighted the growing interest in the development of innovative scaffold designs to overcome often conflict requirements (such as biological and mechanical ones). Considering different pore size gradients, Sobral et al. [21] designed and manufactured. The goal was to enhance cell seeding efficiency and control the spatial organization of cells within the scaffold. Some authors [22] also emphasized the importance of scaffold pore size gradients in osteogenic differentiation of human mesenchymal stromal cells. In 2010, Puppi et al. [23] in deep reviewed the design of biodegradable and bioactive polymeric scaffolds, with properly suited architecture and tailored properties for bone and cartilage tissue regeneration. According to the authors, a good scaffold design must account that macro- and microstructural properties affect cells survival, signaling, growth, propagation, and reorganization and play also a major role in modeling cell shape and gene expressions, both related to cell growth and preservation of native phenotypes [24, 25]. In addition, several scaffold designs were developed and then manufactured using different AM processes. For example, Fierz et al. [26] designed three labeled anisotropic 3D hydroxyapatite scaffolds (pixel-wise and labeled layer-wise) with tailored pores ranging from the nanometer to millimeter scale for the reconstruction of centimeter-sized osseous defects. Seventy percent micrometer-wide pores were successfully interconnected, and virtual spheres (diameter of up to 350 ± 35 μm) were used to simulate cell migration along the pores linked with central channel. Melchels et al. [27] designed poly-DL-lactic acid (PDLLA) porous scaffolds with a gyroid architecture. This architecture was mathematically defined, allowing a precise control of porosity and pore size of a fully interconnected pore network. As noted by the authors, cell seeding of porous structures prepared from hydrophobic polymers, such as PDLLA, was difficult. Moreover, the penetration of a cell suspension was further hindered by the high tortuosity and poor interconnectivity of pore networks when manufactured by salt-leaching or freeze-drying conventional methods. Therefore, very open scaffold structure of the gyroid architecture that facilitates the penetration of water into PDLLA scaffold was manufactured by stereolithography. It was highlighted that the cells were well attached and homogeneously distributed throughout the porous scaffold. Good mechanical properties can be tailored in predesigned (porous) architectures from PDLLA based on gyroid architecture.

In 2012, Melchels et al. [28] reviewed additive manufacturing of tissues and organs. Authors also addressed tailored engineering scaffolds for breast reconstruction, focusing pore size and porosity for the generation of three scaffold models. Cipitria et al. [29] developed a poly (ε-caprolactone) (PCL) scaffold incorporating recombinant human bone morphogenetic protein 7 (rhBMP-7) for the regeneration of critical-sized defects in sheep tibiae. PCL scaffold with b-tricalcium phosphate (mPCL-TCP) to promote bone regeneration was designed based on a honeycomb structure with large interconnected pores to facilitate cellular bridging, ingrowth of bone tissue, and efficient mass transport and vascular infiltration. Moreover, Domingos et al. [30] developed PCL scaffolds for tissue engineering purposes. Authors addressed internal/external scaffold geometry, different material deposition strategies, and the biocompatibility of the material used. 3D PCL porous scaffolds (rectangular porous prisms) were designed with an average porosity of ~76% using commercial computer-aided design software. These structures were then produced via bioextrusion in a 0/90 lay-down pattern trying to reproduce a honeycomb-like pattern of fully interconnected square pores. Similar bioextruded scaffolds were designed (regular dimensions of 600 × 600 mm) to have a well-defined internal geometry with square interconnected pores and uniform distribution. The overall porosity of the structures was found to be ~76%. In vitro degradation of the scaffold was studied as a function of the degradation environment, pore size, and geometry [30, 31]. Scaffold degradation plays a key role when tailoring scaffold properties. In 2016, Morouço et al. [32] developed three types of PCL scaffolds reinforced with cellulose nanofibers (CNF), with and without the addition of hydroxyapatite nanoparticles (HANP), aiming to tailor scaffold properties for tissue engineering applications. The authors studied scaffold porosity, mechanical properties, and biocompatibility as a function of three material combinations. PCL, PCL/CNF, and PCL/CNF/HANP scaffolds were described with porous fully interconnected and porosity (%) of 49.0, 49.5, and 50.0; compressive modulus (MPa) of 54.42, 64.58, and 70.88; and maximum compressive stress (MPa) of 10.96, 11.35, and 12.12, respectively. These structures were then produced via bioextrusion in a 0/90 lay-down pattern. Some authors [33] studied hybrid hierarchical 3D scaffolds with well-controlled architecture for both macro- and microscale. Hybrid and hierarchical 3D structures include thick filaments with the diameter of hundreds of microns, and thin filaments with sub-10 μm dimensions were developed. The microscale features can help in cell seeding, alignment, and guidance. Trying to mimic morphological and mechanical behavior of a blood vessel, Vaz et al. [34] proposed a tailored tissue engineering scaffold. Design parameters such as bilayered tubular scaffold, stiff and oriented outside fibrous layer, and a pliable and randomly oriented fibrous inner layer were considered, combining two biomaterials (PLA/PCL). Structural and mechanical properties of the scaffolds were examined using scanning electron microscopy (SEM) and tensile testing. Cell viability was investigated using 3T3 mouse fibroblasts and the tubular scaffold in an appropriated in vitro environment. The proposed scaffold presented appropriate characteristics to be considered a candidate for blood vessel tissue engineering. Other authors also proposed to tailor tissue engineering scaffolds trying to mimic extracellular matrix morphology of natural tissue for blood vessel applications [35, 36].

In 2012, Chantarapanich et al. [37] developed a computer-aided design library based on polyhedrons for tissue engineering applications. Close-cellular scaffold included truncated octahedron, rhombicuboctahedron, and rhombitruncated cuboctahedron, while open-cellular

scaffold included hexahedron, truncated octahedron, truncated hexahedron, cuboctahedron, rhombicuboctahedron, and rhombitruncated cuboctahedron. Both relationship between pore size and porosity of close-cellular scaffolds and relationship between pore size/beam thickness and porosity of open-cellular scaffolds were studied. The study concluded that some design combinations were not good for making the open-cellular scaffold, generating enclosed pores inside the scaffold, and, therefore, they were excluded from the digital library. Compressive stresses were computed as a function of polyhedron-based geometries which can also be helpful for tailoring mechanical properties of the scaffolds.

In the computer-aided engineering based on tailored scaffold geometry, several digital features should be taken into account to obtain computer-tailored bioengineering scaffolds. Such features encompass cell and growth factor encapsulating, cell aggregation, cell-cell and cell-tissue interaction, vascularization, scaffold degradation (or not if permanent) and tissue growth, drug release, and scaffold mechanical behavior (**Figure 2**). To help digital prediction of cell/tissue phenomena, several automated methods exist, namely, cell counting, cell geometry determination, chromosomal counting, correlation of DNA expression determined through microarrays, interpreting fluorescence data, determining cell's lineage, and cross correlating gene expression with predicted in vivo pathology. All of these features have predictive value for determination of tissue viability and the differentiative rate of cells seeded with the goal of tissue culture. A detailed description about both accumulation of the expression data and large-scale computer cross correlation (between this expression and expressions commonly used in pathology) is provided in Ref. [9], as well as a number of specific tools for tissue analysis/identification.

Despite of the aforementioned research works, new scaffold designs integrating cell/GF/tissue phenomena and scaffold mechanical behavior (geometric characteristics and materials) are needed for regenerative medicine. These complex hierarchical 3D structures must be designed according to the structural heterogeneity of the host tissue and/or scaffold environment.

3. Biomaterials and scaffold bioactivity

Tissues possess different structures and properties that a tissue engineering scaffold should be tailored to. A general requirement for all biomaterial scaffolds is to reproduce an extracellular matrix (ECM) environment for supporting cell growth outside of the body. Moreover, scaffold should host cell adhesion, proliferation, and ECM production. Hence, the scaffold should surrogate the missing ECM. Tissue engineering products can be designed to conduct, induct, or block tissue responses and architectures [38]. Besides providing the three-dimensional growth of cells in an organized way, an ideal scaffold should be characterized by biocompatibility, biodegradability, appropriate mechanical properties, interconnectivity of pores with appropriate size to retain cells, and low exchanges of nutrients and waste products [39]. Tailoring biomaterials for enhanced biofunctionality can be achieved using a variety of approaches that involve the introduction of chemical, topographical, or mechanical cues via top-down or bottom-up approaches [40]. Therefore, the selection of the starting materials and of the fabrication techniques is of paramount importance. Numerous natural and

synthetic materials can be used for the fabrication of scaffolds including polymers, ceramics, bioactive glass, calcium phosphates, and biometals. For example, scaffolds fabricated from bioactive ceramic materials such as hydroxyapatite and tricalcium phosphate show promise because of their biological ability to support bone tissue regeneration. However, the use of ceramics as scaffold materials is limited because of their inherent brittleness and difficult processability [39]. In 2006, Rezwan et al.'s [41] review showed that conventional material processing methods have been adapted and extended for incorporation of inorganic bioactive phases into porous and interconnected 3D polymer networks. The biomaterials were extended from purely synthetic materials to material/biologic hybrids, engineering at the same time bioactivity and biodegradability [41]. Addressing this issues, in 2015, Fiedler et al. [42] focused on the mechanical characterization of PCL-bioglass composites and concluded that the addition of bioglass was found to decrease the elastic gradient and yield stress if two scaffolds of the same density are compared and the highest bioglass content (35%) seems beneficial as it (i) does not significantly deteriorate the scaffold mechanical properties and (ii) promotes bioactivity.

The next generation of synthetic biodegradable, bioactive, living composite biomaterials that feature high adaptiveness to the biological environment [41] considers the incorporation of biomolecules as promising and is currently under extensive research. Incorporating biomolecules such as growth factors during scaffold processing with the aim to accelerate local tissue healing however are not simple as biomolecules are sensitive to elevated temperatures and extreme chemical conditions. A promising strategy is the immobilization of proteins and growth factors in the post-processing phase via surface functionalization of the scaffold [43].

"Soft" material routes like sol-gel processing might be a strategy to incorporate biomolecules during scaffold fabrication. To the authors' knowledge, however, sol-gel-derived bioactive organic/inorganic hybrids have not yet been formed into highly interconnected porous structures, which would be essential for application of these composites as scaffolds. Another related challenge was the elucidation of the local impact of growth factors on the cell and tissue systems, including long-term effects [41]. As pointed out in Section 2, mechanical property is one of the most critical parameters that determine the performance of a designed implant. It mainly depends on the process and structural properties of the biomaterials. Therefore, it is possible to achieve desired mechanical properties through modifying the structural characteristics of a biomaterial. Biological behavior of cell assessment after surface modifications is required to check its biocompatibility and bioactivity [38]. The study of the interactions of biochemical and geometrical cues on stem cell differentiation and alignment should be also considered. The capability to spatially control stem cell orientation and differentiation toward multiple phenotypes simultaneously, i.e., myocyte, tenocyte, and osteoblast, allows cells grown in vitro to more closely mimic aspects of native tissue organization and structure [44]. Although the precise mechanism behind geometry-induced cell alignment is presently unknown, it is likely that the alignment of cells observed on fibers may be attributed to a combination of factors including physical space constraint and relative stiffness of the underlying substrate (fiber); ultimately affecting changes in both cell spreading and cell stiffness, cells may be predisposed toward a specific orientation through the modulation of mechanotransduction pathways via cytoskeletal rearrangements [9].

Multilayer scaffolds and combinations of several biomaterials are a better option to create graded structures that resemble the biological interface. The development of multilayer scaffolds and the controlled release of bioactive molecules to promote in situ regeneration of biological tissues are some of the latest technologies that intended to improve on the available traditional treatments. To confirm the potential of these novel approaches, long-term evaluation is necessary with special focus on studying the biological and mechanical properties of the synthesized tissues [45].

Scaffolds should be designed more as a bioactive system rather than just passive cell carriers. Thus, integration of fabrication techniques with surface modification may also act as route to obtain nanofibrous scaffolds with better understanding of cell scaffolds both in vivo and in vitro. Similarly and significantly, the biomaterial as design strategy can be used in a better way to relate science and engineering, and use this advanced knowledge to engineer more advanced tissue scaffolds [46, 47].

4. Biofabrication processes

Aiming to tailor bioengineering scaffolds that closely mimic the native tissues, AM technologies are suitable to dispense biomaterials (with live cells or cell aggregates) at specific, and desired, locations [48]. The usage of these technologies has been commonly divided in three categories: (i) the jet-based techniques, (ii) robotic dispensing techniques, and (iii) laser-induced forward transfer [49, 50]. Each of these techniques has advantages and drawbacks (**Table 1**). Thus, understanding its limitations and potentials is a must-do to choose the right approach for the specific tissue that is aimed to regenerate. Furthermore, some advancements have been recently achieved with integrated/hybrid systems. These systems combine different techniques within the same equipment aiming to generate a multifunctional graded construct with tailored properties similar to the native tissue.

In the available literature, it is possible to find investigations using different approaches, for the same type of tissue. In fact, there seems to be a trend to some research groups get specialized in some type of technology and use it for various goals.

	Jet based	**Robotic dispensing**	**Laser-induced forward transfer**
Resolution	+	+/–	++
Fabrication speed	+/–	++	–
Hydrogel viscosity	–	+	+/–
Gelation speed	++	+/–	++
Cell density	–	+	+/–

Adapted from Ref. [51].

Table 1. Comparison of the three AM approaches for tissue engineering.

Regarding the jet-based techniques, with a common resolution of 10–50 μm, it is difficult to obtain an adequate structural support. It consists of dispensing a jet of small droplets of liquid material, also called as bioink, in a spatially controlled manner. There are two different approaches, thermal inkjet printing and piezoelectric-actuated inkjet printing, having the former lower suitability for 3D bioengineering scaffolds. Using a piezoelectric actuator, research has been able to suppress some of the thermal constraints [51]. For instance, good viability of printed cell populations was obtained for human fibroblast cell line [52], and recently a silk-based ink eliminated the usage of any cytotoxic organic or inorganic solvents [53]. Even though jet-based techniques are the pioneer techniques used for tissue engineering, translating it to the construct of large 3D structures is a challenge to overcome, mostly because of the low-viscous solutions that do not provide strong and complex 3D structures.

The most successful attempts to engineer cell-containing bioengineering scaffolds have been achieved through robotic dispensing systems. These technologies are based on a controlled extrusion of a material in a continuous fashion, instead of liquid droplets (**Figure 3**) and are developed at the Center for Rapid and Sustainable Development (CDRSP) of the Polytechnic Institute of Leiria, Portugal. Therefore, this approach enables the printing of hydrogels encapsulating cells in a very controlled architecture [28]. The most common methods are the pneumatic [54] or mechanical [55] dispensing systems, comprising (i) a dispensing system and a stage with the capability of moving along the *x*, *y*, and *z* axes; (ii) a light source to illuminate the working area and/or for photoinitiator activation; and (iii) a piezoelectric humidifier [56], with some of them using multiple printing heads to permit the dispensing of various materials without retooling [57]. However, researcher should bear in mind that optimal balance should be aimed between pressure and nozzle size (to obtain higher cell viability).

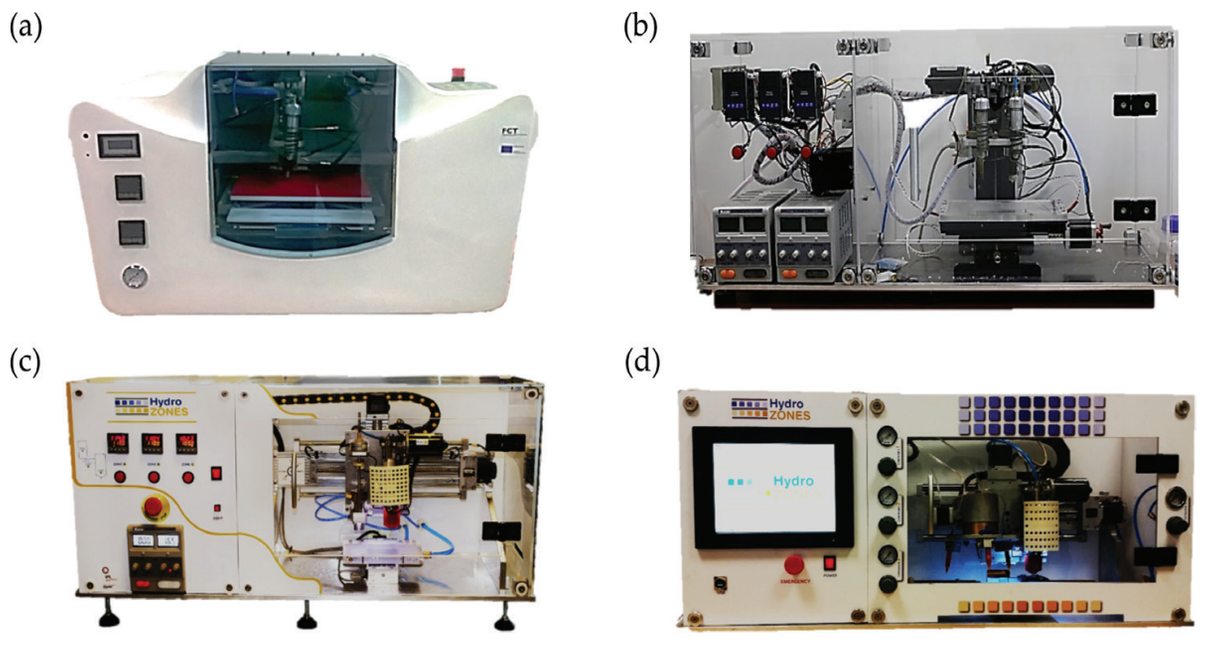

Figure 3. Equipments developed at the CDRSP, Portugal: (a) one-head extrusion system, (b) dual-head extrusion system, (c) system combining an extrusion head and a syringe for hydrogel deposition, and (d) hybrid system combining extrusion with up to three hydrogels.

Recently, a US research group presented an integrated tissue-organ printer (ITOP) for the production of human-scale tissue bioengineering scaffolds of any shape, in a single structure [50]. Combining different procedures, it was possible to successfully engineer (i) a mandible bone, (ii) an ear-shaped cartilage, and (iii) a skeletal muscle. Furthermore, some institutions are now combining different technologies, for instance, merging a robotic dispensing system with a jet-based printing for muscle-tendon unit repair [58].

Lastly, the laser-induced forward transfer (LIFT) is a not-so-common technology for tissue engineering, but is gaining significant importance in this domain [56]. It is based on using three layers of different components: the first layer based on a donor slide, covered by a laser energy-absorbing layer and completed with a cell-bioink component [51]. There are three main advantages for this technology: it is suitable for using (i) a wide range of materials, (ii) a very high precise deposition (but in small 3D structures), and (iii) a clog-free process without the use of nozzles. However, it requires a rapid gelation process, and researchers should bear in mind that several factors should be considered (e.g., laser wavelength, bioink viscosity; for more info read Ref. [59]). Apart from these constraints, successful cell viability (>90%) has been reported for printing skin cell lines and human mesenchymal stem cells and to prepare a cardiac patch [60].

5. Scaffold biodegradability and cell viability

The main objective of tissue engineering is to allow the cells of the body to replace the implanted scaffold over a period. Because bioengineering scaffolds are not intended as permanent implants (besides some of them have shown good results mainly in bone regeneration), they must therefore be biodegradable, so that the need of surgical removal can be avoided. Furthermore, the degradation products should be nontoxic and should be able to swiftly exit from the body without interference with other organs. In addition to this, the intermediate product, the timing of the degradation process, and the route and mechanism of degradation are equally important aspects that need to be taken care [47]. Scaffold materials should fulfill several requirements. A scaffold is not just a passive support for cell growth, but a device whose properties affects the regeneration cascade. Mechanical properties, surface properties, and morphology are in turn relevant to the specific application. Degradation kinetics and the rate at which scaffold properties change with degradation should always be predictable. In particular, the degradation behavior of biomaterials can follow several mechanisms and is controlled by different factors. Understanding the degradation kinetics and mechanism of biomaterials is necessary to optimize their possible usage. The rate of degradation is also strictly connected to the degree of porosity [38].

One of the general variables that need to be thoroughly considered to successfully bioprint viable and functional tissue bioengineering scaffolds is the inclusion of supportive biomaterials, generally in the form of proteins and polymers, which (1) facilitate the deposition method by mechanical means and (2) provide support and protection to the cells during and after the

tissue construct fabrication process. These biomaterials can encompass the physical environment inside of which the cells will reside, as well as the biochemical signal cells need to function as they would in the body [46].

Scaffolds represent the space available for the tissue to develop and the physical support for cell growth. Scaffold mechanical properties should allow shape maintenance during tissue regeneration and enable stress transfer and load bearing. Moreover, during the first stage of tissue reconstruction, wound contraction forces act against the process, and enough mechanical strength and stiffness of the scaffold is required. Scaffold porosity is a fundamental characteristic for providing available space for cells to migrate and for vascularization of the tissue. Furthermore, the larger the surface available, the more cell interactions will arise. In general, the biological activity of a scaffold is determined by ligand density. Scaffold composition and porous fraction, that is, the total surface of the structure exposed to cells, determine the ligand density. Highly specific surface areas allow for cell attachment and anchorage, and a high pore volume fraction enables cell growth, migration, and effective transportation of fluids and nutrients. In particular, microporosity is important for capillary ingrowth and interactions between cells and matrix, while macroporosity is relevant to nutrient supply and waste removal of cell metabolism. The rate of degradation is also strictly connected to the degree of porosity [38].

As in the development of the tissue-engineered organs, regeneration of functional tissue requires maintenance of cell viability and differentiated function, encouragement of cell proliferation, modulation of the direction and speed of cell migration, and regulation of cellular adhesion [61]. Cell viability may be judged by morphological changes or by changes in membrane permeability and/or physiological state inferred from the exclusion of certain dyes or the uptake and retention of others. Cultured cells are seeded onto a three-dimensional biocompatible scaffold that will slowly degrade and resorb as the soft and hard structures grow and assimilate in vitro and/or in vivo [2]. Cell viability during 3D bioprinting is dependent on the shear stress experienced during extrusion, which in turn is dependent on the viscosity of the solution, the applied pressure, and the needle diameter. In addition, any post-printing bioink cross-linking may also impact on cell viability [62]. Cell viability can be measured with Live/Dead Viability/Cytotoxicity assay after printing [63] and could vary with dispensing pressure and nozzle diameter. It decreases as the pressure increases and the nozzle diameter decreases, and it is seen that the effect of pressure is significantly larger than the effect of the nozzle diameter. At higher pressures, there is an increase in the number of apoptotic cells as well as necrotic cells [64].

Tissue bioengineered scaffolds targeted for in vivo applications are typically restricted to a thickness of only a few hundreds of microns, owing to the diffusion limitations of oxygen and nutrients [43]. One of the major challenges in tissue engineering for translation in clinical applications is the vascularization of bioengineering scaffolds of clinically relevant size. Insufficient vascularization inhibits nutrient and host cell delivery or migrations and leads to improper cell integration or cell death. While vascularization remains a challenge to maintain viability of large biofabricated tissue bioengineering scaffolds, recent advances in the field demonstrate that novel biofabrication techniques may resolve this problem [65].

6. New insights: 3D to 4D

Doing a survey on the Web of Science®, it is noticeable that the number of original articles on tissue engineering and regenerative medicine has experienced a tremendous increase over the past 10 years (**Figure 4**; review papers and proceedings not included). Likewise, bioprinting is attracting a lot of researchers presenting an exponential increase in the last 3 years. Meanwhile, 3D bioprinting market was valued at $98.6 million in 2015, and an annual growth of 36% for the next 6 years is expected [66].

Nevertheless, 3D bioprinting has been focused on the development of bioengineering scaffolds that lack a crucial element for mimicking native live tissues: its ability to acutely change according to its function. That is why leading research groups have recently proposed the four-dimensional (4D) bioprinting (time is integrated with 3D bioprinting) as an enhanced approach for tissue engineering and regenerative medicine: the development of stimuli-responsive biomaterials that can be printed and dynamic to intended stimulation. However, several challenges arise, namely, (i) bioinks have to be optimized to achieve successful bioprinting; (ii) processes must be mechanically designed to obtain robust shape-changing capability of the bioengineering scaffolds [67]; (iii) specific bioreactors for complex tissue function maturation need to be invented; and (iv) evaluation procedures should be defined to examine the functionality response.

Therefore, the most promising approach is to optimize the cell-bioengineering scaffold interactions, becoming feasible to explore the usage of computer modeling to examine the further responses. Developing "smart" biomaterials (also referred as "intelligent," "stimuli responsive," "stimuli sensitive," or "environmentally sensitive") to allow the dynamic changes of the structure, upgrade of the printing processes into defined architecture for targeting tissues,

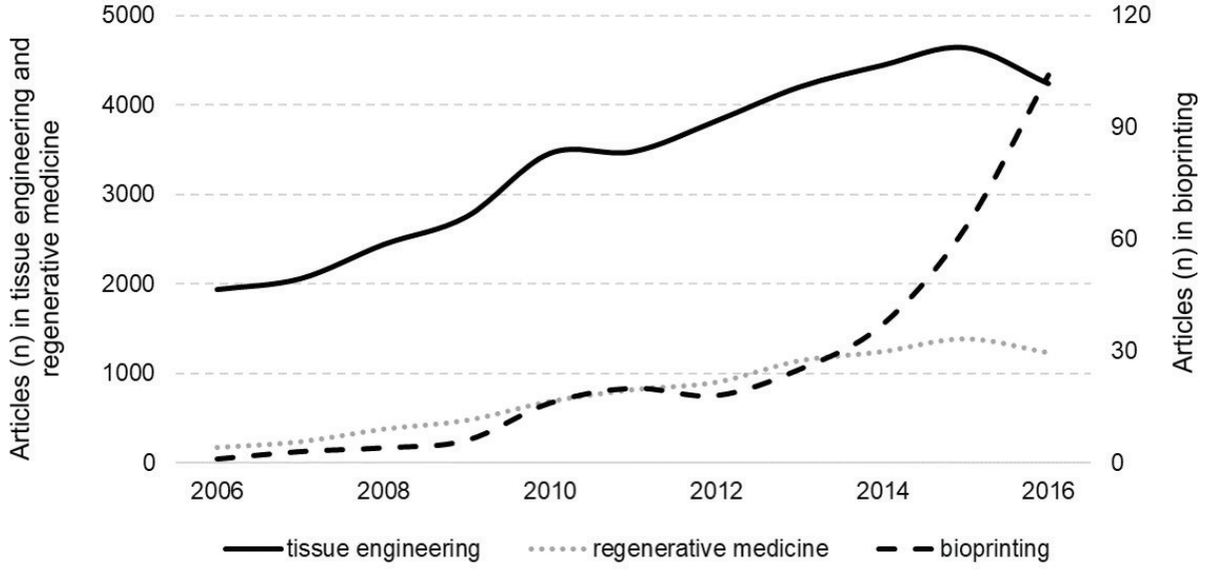

Figure 4. Number of original articles on tissue engineering and regenerative medicine 2006–2016.

automation of stimulus, and standardizing the assessment procedures to evaluate the result is crucial for enhanced regenerative medicine approaches [68].

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References

[1] Morouço P, Alves N, Amado S. The role of biomechanics in tissue engineering. Austin Journal of Biomedical Engineering. 2016;**3**(1):1-2

[2] Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues—state of the art and future perspectives. Journal of Biomaterials Science Polymer Edition. 2001;**12**(1):107-124

[3] Malda J, Groll J. A step towards clinical translation of biofabrication. Trends in Biotechnology. 2016;**34**(5):356-357

[4] Zadpoor AA, Malda J. Additive manufacturing of biomaterials, tissues, and organs. Annals of Biomedical Engineering. 2017;**45**(1):1-11

[5] Parker GJM, Wheeler-Kingshott CAM, Barker GJ. Diffusion tensor imaging. IEEE Transactions on Medical Imaging. 2002;**21**(5):505-512

[6] Fang Z, Starly B, Sun W. Computer-aided characterization for effective mechanical properties of porous tissue scaffolds. CAD Computer-Aided Design. 2005;**37**(1):65-72

[7] Zadpoor A.A.. Bone tissue regeneration: The role of scaffold geometry. Biomaterials Science. 2015;**3**(2):231-245

[8] Folch A, Mezzour S, Düring M, Hurtado O, Toner M, Müller R. Stacks of microfabricated structures as scaffolds for cell culture and tissue engineering. Biomedical Microdevices. 2000;**2**(3):207-214

[9] Sun W, Darling A, Starly B, Nam J. Computer-aided tissue engineering: Overview, scope and challenges. Biotechnology and Applied Biochemistry. 2004;**39**(Pt 1):29-47

[10] Landers R, Hubner U, Schmelzeisen R, Mulhaupt R. Rapid prototyping of scaffolds derived from thermoreversible hydrogels and tailored for applications in tissue engineering. Biomaterials. 2002;**23**(23):4437-4447

[11] Lin A, Barrows T, Cartmell S, Guldberg R. Microarchitectural and mechanical characterization of oriented porous polymer scaffolds. Biomaterials. 2003;**24**(3):481-489

[12] Angelo DF, Morouço P, Alves N, Viana T, Santos F, González R, et al. Choosing sheep (*Ovis aries*) as animal model for temporomandibular joint research: Morphological, histological and biomechanical characterization of the joint disc. Morphologie. 2016; **100**(331):223-233

[13] Wieding J, Souffrant R, Mittelmeier W, Bader R. Finite element analysis on the biomechanical stability of open porous titanium scaffolds for large segmental bone defects under physiological load conditions. Medical Engineering and Physics. 2013;**35**(4):422-432

[14] Cheng XY, Li SJ, Murr LE, Zhang ZB, Hao YL, Yang R, et al. Compression deformation behavior of Ti-6Al-4V alloy with cellular structures fabricated by electron beam melting. Journal of the Mechanical Behavior of Biomedical Materials. 2012;**16**(1):153-162

[15] Van Der Stok J, Koolen MKE, De Maat MPM, Amin Yavari S, Alblas J, Patka P, et al. Full regeneration of segmental bone defects using porous titanium implants loaded with BMP-2 containing fibrin gels. European Cells & Materials. 2015;**29**:141-154

[16] van der Stok J, Wang H, Amin Yavari S, Siebelt M, Sandker M, Waarsing JH, et al. Enhanced bone regeneration of cortical segmental bone defects using porous titanium scaffolds incorporated with colloidal gelatin gels for time- and dose-controlled delivery of dual growth factors. Tissue Engineering Part A. 2013;**19**(23-24):2605-2614

[17] Van Bael S, Chai YC, Truscello S, Moesen M, Kerckhofs G, Van Oosterwyck H, et al. The effect of pore geometry on the in vitro biological behavior of human periosteum-derived cells seeded on selective laser-melted Ti6Al4V bone scaffolds. Acta Biomaterialia. 2012;**8**(7):2824-2834

[18] Hollister SJ. Porous scaffold design for tissue engineering. Nature Materials. 2005 Jul;**4**(7):518-524

[19] Khoda KM, Ozbolat IT, Koc B. A functionally gradient variational porosity architecture for hollowed scaffolds fabrication. Biofabrication. 2011;**3**(3):34106

[20] Giannitelli S, Mozetic P, Trombetta M, Rainer A. Combined additive manufacturing approaches in tissue engineering. Acta Biomaterialia. 2015;**24**:1-11

[21] Sobral JM, Caridade SG, Sousa RA, Mano JF, Reis RL. Three-dimensional plotted scaffolds with controlled pore size gradients: Effect of scaffold geometry on mechanical performance and cell seeding efficiency. Acta Biomaterialia. 2011;**7**(3):1009-1018

[22] Di Luca A, Szlazak K, Lorenzo-Moldero I, Ghebes CA, Lepedda A, Swieszkowski W, et al. Influencing chondrogenic differentiation of human mesenchymal stromal cells in scaffolds displaying a structural gradient in pore size. Acta Biomaterialia. 2016;**36**(March):210-219

[23] Puppi D, Chiellini F, Piras MM, Chiellini E. Polymeric materials for bone and cartilage repair. Progress in Polymer Science. 2010;**35**(4):403-440

[24] Chua C, Leong K, Lim C. Classification of rapid prototyping systems. In: Chua C, Leong K, Lim C, editors. Rapid Prototyping: Principles and Applications. Singapore: World Scientific Publishing; 2003. pp. 19-23

[25] Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials. 2005;**26**:5474-5491

[26] Fierz FC, Beckmann F, Huser M, Irsen SH, Leukers B, Witte F, et al. The morphology of anisotropic 3D-printed hydroxyapatite scaffolds. Biomaterials. 2008;**29**(28):3799-3806

[27] Melchels FPW, Feijen J, Grijpma DW. A poly(d,l-lactide) resin for the preparation of tissue engineering scaffolds by stereolithography. Biomaterials. 2009;**30**(23-24):3801-3809

[28] Melchels FPW, Domingos MAN, Klein TJ, Malda J, Bartolo PJ, Hutmacher DW. Additive manufacturing of tissues and organs. Progress in Polymer Science. 2012;**37**(8):1079-1104

[29] Cipitria A, Reichert JC, Epari DR, Saifzadeh S, Berner A, Schell H, et al. Polycaprolactone scaffold and reduced rhBMP-7 dose for the regeneration of critical-sized defects in sheep tibiae. Biomaterials. 2013;**34**(38):9960-9968

[30] Domingos M, Dinucci D, Cometa S, Alderighi M, Bártolo PJ, Chiellini F. Polycaprolactone scaffolds fabricated via bioextrusion for tissue engineering applications. International Journal of Biomaterials. 2009;**2009**:239643

[31] Domingos M, Chiellini F, Cometa S, De Giglio E, Grillo-Fernandes E, Bártolo P, et al. Evaluation of in vitro degradation of PCL scaffolds fabricated via BioExtrusion. Part 1: Influence of the degradation environment. Virtual and Physical Prototyping. 2010;**5**(2):65-73. Available from: http://www.tandfonline.com/doi/abs/10.1080/17452751003769440

[32] Morouço P, Biscaia S, Viana T, Franco M, Malça C, Mateus A, et al. Fabrication of poly(ε-caprolactone) scaffolds reinforced with cellulose nanofibers, with and without the addition of hydroxyapatite nanoparticles. BioMed Research International. 2016;**2016**:1-10

[33] Wei C, Dong J. Hybrid hierarchical fabrication of three-dimensional scaffolds. Journal of Manufacturing Processes. 2014;**16**(2):257-263

[34] Vaz CM, van Tuijl S, Bouten CVC, Baaijens FPT. Design of scaffolds for blood vessel tissue engineering using a multi-layering electrospinning technique. Acta Biomaterialia. 2005;**1**(5):575-582

[35] Nemeno-Guanzon JG, Lee S, Berg JR, Jo YH, Yeo JE, Nam BM, et al. Trends in tissue engineering for blood vessels. Journal of Biomedicine and Biotechnology. 2012;**2012**

[36] Hoenig MR, Campbell GR, Rolfe BE, Campbell JH. Tissue-engineered blood vessels: Alternative to autologous grafts? Arteriosclerosis, Thrombosis, and Vascular Biology. 2005;**25**(6):1128-1134

[37] Chantarapanich N, Puttawibul P, Sucharitpwatskul S, Jeamwatthanachai P, Inglam S, Sitthiseripratip K. Scaffold library for tissue engineering: A geometric evaluation. Computational and Mathematical Methods in Medicine. 2012;**2012**

[38] Haycock J. 3D Cell Culture [Internet]. Vol. 695, Image (Rochester, N.Y.). 2011. pp. 261-280

[39] Seol YJ, Park DY, Park JY, Kim SW, Park SJ, Cho DW. A new method of fabricating robust freeform 3D ceramic scaffolds for bone tissue regeneration. Biotechnology and Bioengineering. 2013;**110**(5):1444-1455

[40] Camarero-Espinosa S, Cooper-White J. Tailoring biomaterial scaffolds for osteochondral repair. International Journal of Pharmaceutics. 2016;**523**(2): 476-489

[41] Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials. 2006;**27**(18):3413-3431

[42] Fiedler T, Videira AC, Bártolo P, Strauch M, Murch GE, Ferreira JMF. On the mechanical properties of PLC-bioactive glass scaffolds fabricated via BioExtrusion. Materials Science and Engineering C. 2015;**57**:288-293

[43] Cosson S, Otte EA, Hezaveh H, Cooper-White JJ. Concise review: Tailoring bioengineered scaffolds for stem cell applications in tissue engineering and regenerative medicine. Stem Cells Translational Medicine. 2015;**4**(2):156-164

[44] Ker EDF, Nain AS, Weiss LE, Wang J, Suhan J, Amon CH, et al. Bioprinting of growth factors onto aligned sub-micron fibrous scaffolds for simultaneous control of cell differentiation and alignment. Biomaterials. 2011;**32**(32):8097-8107

[45] López-Ruiz E, Jiménez G, García MÁ, Antich C, Boulaiz H, Marchal JA, et al. Polymers, scaffolds and bioactive molecules with therapeutic properties in osteochondral pathologies: What's new? Expert Opinion on Therapeutic Patents. 2016;**26**(8):877-890

[46] Cheung DYC, Duan B, Butcher JT. Essentials of 3D Biofabrication and Translation [Internet]. Essentials of 3D Biofabrication and Translation. 2015. pp. 351-370

[47] Kariduraganavar MY. Advances in polymers and tissue engineering scaffolds. In: Inamuddin, editor. Green Polymer Composites Technology Properties and Applications. Boca Raton: Taylor & Francis Group; 2016;343-354

[48] Gao B, Yang Q, Zhao X, Jin G, Ma Y, Xu F. 4D bioprinting for biomedical applications. Trends in Biotechnology. 2016;**34**(9):746-756

[49] Melchels F, Malda J, Fedorovich N, Alblas J, Woodfield T. Organ Printing; Comprehension Biomaterials; 2011;**5**:587-606

[50] Kang H-W, Lee SJ, Ko IK, Kengla C, Yoo JJ, Atala A. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. Nature Biotechnology. 2016;**34**(3):312-319

[51] Malda J, Visser J, Melchels F, JÜngst T, Hennink W, Dhert W, et al. 25th anniversary article: Engineering hydrogels for biofabrication. Advanced Materials. 2013;**25**:5011-5028

[52] Saunders R, Gough J, Derby B. Delivery of human fibroblast cells by piezoelectric drop-on-demand inkjet printing. Biomaterials. 2008;**29**:193-203

[53] Tao H, Marelli B, Yang M, An B, Onses M, Rogers J, et al. Inkjet printing of regenerated silk fibroin: From printable forms to printable functions. Advanced Materials. 2015;**27**:4273-4279

[54] Chang C, Boland E, Williams S, Hoying J. Direct- write bioprinting three-dimensional biohybrid systems for future regenerative therapies. Journal of Biomedical Materials Research. Part B, Applied Biomaterials. 2011;**98**:160-170

[55] Visser J, Peters B, Burger T, Boomstra J, Dhert W, Melchels F, et al. Biofabrication of multi-material anatomically shaped tissue constructs. Biofabrication. 2013;**5**(3):35007

[56] Murphy S, Atala A. 3D bioprinting of tissues and organs. Nature Biotechnology. 2014; Aug;**32**(8):773-785

[57] Mironov V, Visconti R, Kasyanov V, Forgacs G, Drake C, Markwald R. Organ printing: Tissue spheroids as building blocks. Biomaterials 2009;**30**:2164-2174

[58] Merceron T, Burt M, Seol Y, Kang H, Lee S, Yoo J, et al. A 3D bioprinted complex structure for engineering the muscle-tendon unit. Biofabrication. 2015;**7**:35003

[59] Guillemot F, Souquet A, Catros S, Guillotin B. Laser- assisted cell printing: Principle, physical parameters versus cell fate and perspectives in tissue engineering. Nanomedicine. 2010;**5**:507-515

[60] Gaebel R, Ma N, Liu J, Guan J, Koch L, Klopsch C, et al. Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration. Biomaterials. 2011;**32**:9218-9230

[61] Khang G. Handbook of Intelligent Scaffolds for Tissue Engineering and Regenerative Medicine; Singapore: Taylor & Francis Group; 2012. 1-972 pp

[62] Daly AC, Critchley SE, Rencsok EM, Kelly DJ. A comparison of different bioinks for 3D bioprinting of fibrocartilage and hyaline cartilage. Biofabrication. 2016;**8**(4):45002

[63] Cui X, Breitenkamp K, Finn MG, Lotz M, D'lima DD. Direct human cartilage repair using three-dimensional bioprinting technology. Tissue Engineering Part A. 2012;**18**:1304-1312

[64] Nair K, Gandhi M, Khalil S, Yan KC, Marcolongo M, Barbee K, et al. Characterization of cell viability during bioprinting processes. Biotechnology Journal. 2009;**4**(8):1168-1177

[65] Lim K, Morouço P, Levato R, Melchels F, Malda J. Organ Biofabrication. Comprehension Biomaterials II; 2017;**5**:236-266

[66] Market Research P&S. Global 3D Bioprinting Market Size, Share, Development, Growth and Demand Forecast to 2022, 2016. Available from (https://www.psmarketresearch.com/press-release/3d-bioprinting-market)

[67] Li Y-C, Zhang YS, Akpek A, Shin SR, Khademhosseini A. 4D bioprinting: The next-generation technology for biofabrication enabled by stimuli-responsive materials. Biofabrication. 2016 2;**9**(1):012001

[68] Furth ME, Atala A, Van Dyke ME. Smart biomaterials design for tissue engineering and regenerative medicine. Biomaterials. 2007;**28**(34):5068-5073