Chapter

Comprehensive Review on Full Bone Regeneration through 3D Printing Approaches

Cristiana Fernandes, Carla Moura, Rita M.T. Ascenso, Sandra Amado, Nuno Alves and Paula Pascoal-Faria

Abstract

Over the last decades, the number of work accidents associated with bone fractures has increased leading to a growing concern worldwide. Currently, autografts, allografts, and xenografts are used for bone regeneration. However, their application has associated risks. Tissue engineering (TE) has brought solutions to address these problems, through the production of temporary supports, providing mechanical support to the formation of new bone tissue and biocompatible and biodegradable scaffolds, which allow cell adhesion and proliferation to ensure bone formation. The combination of materials and structure with the technique to be used will directly influence their physical and chemical properties and, consequently, their action in contributing to bone regeneration. Thus, the focus of this chapter is to perform an exhaustive literature review and a critical analysis of the state of the art in bone TE and present a proposal of an optimized temporary support geometry for bone regeneration in case of large bone defects. For this, it was listed and identified the best choice of biomaterials, fabrication method, cell type and their culture conditions (static vs. dynamic), and/or the inclusion of growth factors for the repair of large bone defects.

Keywords: large bone defects, bone regeneration, tissue engineering, cell culture, CAD, scaffolds, additive manufacturing

1. Introduction

The population is increasingly exposed to accidents, both in daily routine and at work. In Portugal, among 209,390 non-death accidents that occurred in 2017, almost 4% were bone fractures that are limitative for the active population and require a long time of recovery [1]. Many research groups have been working on bone regeneration for over 10 years, but this has not led to effective therapy in a clinical setting. If it was successful, it would enhance the quality of life for millions of people and significantly reduce the absence to work due to fractures which are considered the second higher cause of working day lost.

The bone is a natural composite containing organic components (mainly collagen type I and fibrillin) and inorganic crystalline minerals (such as hydroxyapatite (Hap)), defined as hard tissue [2–4]. The characteristic of the collagen fibers in

their structure gives it high tensile strength and its mineral substances impart high compressive strength and thus excellent mechanical resistance.

Bone, namely in the diaphysis, is made up of cortical or compact bone that contains its own blood vessels and cells, which aid in its growth and regeneration. It has many types of cells, such as osteoblasts, osteocytes, osteoclasts, and a matrix of non-mineralized collagen (osteoid). Bone tissue comprises several functions, such as: (i) provide structural integrity, and all the necessary support to the soft tissue of the body, constituting the global support of the majority of the muscles, (ii) protect vital organs, and (iii) help to balance the minerals, since the bone tissue stores calcium and phosphate making them more resistant and able to maintain a balance of blood concentration [5–7]. Bone is known to self-regenerate: pos-natal bone maintains an intrinsic capacity for well-ordered growth, remodeling to meet mechanical needs, and renewal after damage [8].

Major bone defects are the result of injury, trauma, nonunion after a fracture, infection, or abnormality, resulting in long-term deformities, such as limb shortening, leaving patients with reduced bone structure and function [9–11]. It should be noted that the most transplanted tissue after blood is bone [12, 13].

The gold standard treatments for bone defects are still bone grafts. These can be used alone or combined with other materials in order to promote bone healing through osteoinduction, osteoconduction, and osteogenesis [14]. These bone grafts may be from autograft (taken from the patient), allograft (taken from another patient) and xenograft (obtained from an animal) origins or even manipulated with synthetic biomaterials. Additionally, prostheses can also be used, but they usually need a second surgery later on due to some complications that may appear, such as the formation of bone callus or hernias. Autografts are still considered the gold standard treatment due to their osteogenic, osteoinductive, and osteoconductive capacity. However, there is a limitation in tissue extraction from the amount that is required. Allografts taken from other donors or corpses present a high risk of immune rejection, reduced bioactivity and a high risk of pathogen transmission [7, 15–18].

When there are fractures with a bone defect exceeding a critical size, the bone is not able to self-regenerate and, therefore, requires the use of a temporary implant (natural and/or synthetic) to serve as support and cells to help bone regeneration [19]. In this way, tissue engineering (TE) has emerged [20].

The concept of TE was implemented in 1993 by Langer and Vacanti. They specified that "TE is an interdisciplinary field that relates the principles of biology and engineering to the production of tissue functional substitutes" [21]. So, they presented specific characteristics and applications in biodegradable three-dimensional (3D) scaffolds. Ideally, they should be highly porous, having highly interconnected pore networks with a pore size suitable for cells to migrate and differentiate whenever necessary [22]. However, the biggest challenge of scaffolds is related to mass transport of nutrients and secretion of waste in tissue [6]. It is important cells used in 3D cultures of scaffolds be able to mimic the morphology, functionality, and biology of the tissue. These cell cultures are necessary to analyze mechanisms of chronic diseases and the impact of drug treatments or to produce different tissues for major defects in vivo, in this study, the bone. Bioreactors appeared to improve the field of cell culture on 3D support [23, 24].

This chapter intends to perform a critical analysis of the state of the art regarding full bone TE towards the selection of the most appropriate solution of temporary implants. Thus, the optimum conditions (static vs. dynamic), material, cells, and/or the inclusion of growth factors for the repair of large bone defects are discussed. Hence, there are two scientific questions to which this chapter intends to address: (i) which is the most suitable combination of scaffold design and

fabrication using a certain biomaterial and biological components to facilitate or accelerate bone regeneration and (ii) what are the in vitro conditions more suitable to achieve an optimized in vivo response.

2. The usefulness of temporary implants for bone regeneration

Bone tissue is known for its ability to self-regenerate on its own. However, if the fracture becomes a critical bone defect, the bone loses this ability. From 1934 to the present day, some authors argue that a bone defect becomes critical when it is over two times the diameter of the bone defect [10, 11, 25–27].

These critical-sized defects may result from infection, malformation, and traumatic injuries, which may lead to bone loss in the patient [28–33]. In this case, as bone cannot self-regenerate, it is necessary to use a temporary implant (natural and/or synthetic) to support bone regeneration with cellular incorporation. To achieve this, successfully, it is first necessary to consider the mechanical properties of the native bone tissue.

It is known that the mechanical properties of the bone vary according to age, anatomical location, and bone quality. Within the biomechanical properties of the bone (resistance, stiffness, and fatigue), the elastic modulus is the most attracting variable in research due to its importance to characterize bone pathologies and also in the design orientation of artificial implants. Bone strength and elasticity are anisotropic. The compact bone is stronger under compression and stiffer when loaded longitudinally along the diaphyseal axis than in the transverse radial directions. In trabecular bone, its mechanical properties depend on both the porosity and the architectural desirability of the individual trabeculae [3]. The mechanical properties of human bone are summarized in **Table 1**.

Implants need to be accepted by the human body, where there are guarantees for cell survival in a safe and supportive environment. Moreover, mechanical damage or failure caused by stress shielding must be prevented. The scaffolds need to have an appropriate modulus of elasticity to match bone properties. Scaffolds with a highly porous structure are favorable for cellular activities, including fixation and proliferation, which will contribute to bone neoformation and regeneration and adjust the mechanical properties in terms of Young's strength and modulus [36].

So, an important key factor is concerned with the type of materials to be used in the implant. Biopolymers are biocompatible and biologically active materials as they promote cell adhesion and growth.

To help in the engineering of long bone fracture regeneration, artificial fractures are typically manufactured in models in vivo [23, 37]. Various animal models are studied in vivo before its application in humans.

Human bone	**Trabecular**	**Cortical**
Porosity (%)	50.00–90.00	1.00–20.00
Young's modulus E (GPa)	0.05–0.10	17.00–20.00
Compressive strength (MPa)	5.00–10.00	131.00–224.00
Tensile strength (MPa)	1.50–38.00	35.00–283.00
Elongation at break (%)	0.50–3.00	1.07–2.10
References	[2, 3, 34, 35]	

Table 1.
Human long bone properties.

3. In vivo studies

The bone, in vivo, is exposed to mechanical stimulation by muscle contraction and body movements, and the mechanical load induces an increase in bone mass formation [38]. During body movement, the forces applied results in changes in hydrostatic pressure, fluid flow-induced shear stress, direct cell strain, and electric fields [38–40].

In order to identify the mechanical properties necessary for humans, it is important to study what kind of in vivo studies and which animal models have been considered in the literature (see **Table 1**). The choice of the most appropriate animal model is an important step in clinical translation, because it will help to better understand and propose innovative strategies for bone regeneration. Each animal model has pros and cons [41], and in each study, a specific set of parameters is used. That is why it is difficult to compare the different studies available [42, 43]. There are various models that were studied in vivo for full bone regeneration. Rabbit, rat, ovine (sheep or bovine), canine, and goat are the most used.

There are six studies on the literature where a rabbit model was considered. Nather and their co-workers [44] evaluated the effect of bone marrow mesenchymal stem cells (BMMSCs) on the biological healing of a 1.5 cm cortical bone allograft in the tibia of adult rabbits. In their study it was shown that BMMSCs can improve cortical allograft binding rate, reabsorption activity, bone formation, and osteocyte cell count. In 2013, Khojasteh et al. [45] developed a scaffold using particulate mineralized bone/fibrin glue/mesenchymal stem cells (MSCs). Through the alizarin staining method, they verified that there was a deposition of mineralized matrix. This was also demonstrated by RT-PCR analysis of osteocyte markers. At the end of 3 weeks, osteocalcin, osteopontin, and collagen I messenger RNA were produced. They concluded that this implant would be a promising combination for vertical bone augmentation around implants inserted simultaneously into the tibia of rabbits. Lee et al. [46] studied the effect of autologous BMMSCs seeded into gel foam on structural bone allograft healing in 1.5 cm femoral defect of white rabbits. They concluded that the use of MSCs influenced the bone formation, resorption, and angiogenesis. Jang et al. [47] extruded porous HAp scaffolds, which were set in a drill-cut femur rabbit bone. After 4 and 8 weeks of implantation, micro-CT scanning images showed material degradation and integration of the sample into the native bone. In this period, the morphological behavior was similar in bone tissue-scaffold junction. Chowdhary et al. [48] had evaluated the early response of bone tissue to micro threads with an oxidized titanium implant (4 mm in diameter and 8 mm in length) between the macro threads. The study was tested in rabbit legs, tibia, and femur. The bone regeneration happened near the micro threads, and the bone growth in femur indicated that the cancellous bone seems to be more sensitive to micro thread stimulation. Recently, in 2018, Tovar and co-workers [49] used 3D printing, specifically robocasting/direct writing, to develop a scaffold with 100% beta-tricalcium phosphate (β-TCP) (350 μm pore diameter) in order to regenerate critical-sized rabbit radius defects in vivo. A 3 cm incision was made in a critical defect of 11 mm, approximately, in the radio, and the periosteum was resected to at least 1.5 cm proximal and distal to the defect. This scaffold proved to be good for bone tissue engineering (BTE) since at 8 weeks it showed bone formation with signs of resorption of the scaffold. The amount of bone formed was increased from week to week, regenerating the medullary space, and at 24 weeks the scaffold was significantly resorbed.

Rats are also another in vivo model referred in the literature. Saravanan et al. [50] introduced in an albino-Wistar rat with a critical-sized bone defect in the tibia a scaffold containing chitosan, gelatin, and graphene oxide by freeze drying.

They concluded that this scaffold promoted increase in osteoblasts and increased the collagen content, accelerating the bridging of the rat tibial bone defect.

A model of an ovine with 35 mm tibial defect was then used by Smith et al. [51] to study full bone regeneration. They produced a scaffold using blending process of poly (L-lactic acid)-poly(ε-caprolactone) (PLLA: PCL 20:80) with and without marrow-derived skeletal stem cells. They divided the tests into three different groups: empty defect, scaffold alone, and scaffold with cells. Radiographic has shown poor bone formation upon 12 weeks. However, there was a greater tendency for bone formation in the scaffold with cells.

Regarding the canine model, two studies were published in the literature. In 1996, Bragdon and co-workers [52] showed that, in canine femurs, an oscillating motion of 20 μm does not affect osseointegration. However, 40 and 150 μm oscillating motion of implants does not support bone growth. Recently, Barba et al. [19] implanted in vivo, in a canine model calcium-deficient scaffold (0.3 mm height and 5 mm diameter) with Hap considering different pore architectures and compared with two ceramics, a biphasic calcium phosphate (BCP) and a β-TCP with rat mesenchymal stem cells (rMSCs). Pores ranged from 10 to 300 μm. With this, calcium-deficient scaffold and Hap triggered osteogenic differentiation of rMSCs. They concluded that calcium-deficient HAp foam scaffolds with a spherical concave macroporosity allow osteoinduction.

Animal studies are needed to understand bone regeneration. Variables such as the amount of bone formation and its kinetics, mechanical properties and safety obtained by the scaffold, including the presence of toxic degradation in different organs and in terms of inflammatory response need to be understood in detail [42]. However, bone fractures performed in animals do not represent the complexity of healing human fractures [23, 37]. The potential of each different type of cells both in vitro and in vivo plays here a key role.

Ko and co-workers studied the potential of human-induced pluripotent stem cells (hiPSCs) against the human bone marrow mesenchymal stem cells (hBMMSCs). Both cells were placed in rat bone defects, with a size of 2 cm, which is similar to the human value mentioned above. They concluded that both hIPSCs and hBMMSCs have osteogenic potential in vivo [53]. However, some authors showed the existence of risks of teratoma formation after transplantation in hIPSCs [28, 54–56]. In literature, the use of MSCs seems to significantly help bone regeneration in in vivo studies [31, 45, 57–59]. Some authors defend that the addition of growth factors to cell-scaffold constructs promotes bone regeneration [60]. Nevertheless, Kleinhans et al. [61] showed that a good culture capable of mimicking tissue morphology, functionality, and biology, for example, using bioreactors, is sufficient to obtain a homogeneous cell distribution of soluble factors.

There is a great deal of discussion today about the incorporation of growth factors. In this chapter, authors defend the nonnecessity of its incorporation, since upon the right environmental conditions, cells are actually able to secrete the optimal extracellular matrix (ECM) components. Therefore, a good mechanically stimulated culture combined with transcription factors influences cells to bone formation.

4. In vitro studies

In vitro models are required to accurately record the physiology of healing at a site of bone fracture since bone takes weeks to differentiate in vitro [62] and wound healing can take weeks to months [23, 63]. In vitro studies are advantageous because they offer a controlled environment to experimental test molecular and cellular

hypotheses. However, cells cultured in vitro are not replicates of their in vivo counterparts [28, 64].

When the bone is subjected to a mechanical force, electrical potentials are generated, which play an important role in bone remodeling. To mimic this natural process, bioreactors were created and are nowadays widely used. These 3D systems allow the control of various parameters, such as temperature, pH, oxygen concentration, growth factors, and mechanical stimuli, among others, and modulate cell growth more easily. These bioreactors can simulate the human bone environment and allow the study of the role of various factors in scaffolds or preculture scaffolds in vivo. In addition, to provide adequate nutrition and removing residues from all cells in the scaffold, fluid flow can be manipulated to physically stimulate bone growth [39]. Bone is constantly exposed to mechanical stimulation due to muscle contractions and body movements that result in changes in hydrostatic pressure, direct cell strain, fluid flow-induced shear stress, and electric fields. In addition, bone cells are more sensitive to mechanical stimulation. Therefore, providing physical stimulation in bioreactors becomes a key component of BTE strategies [65].

The following studies demonstrate the importance of performing in vitro testing in order to find the best strategy.

Jang et al. [66] developed a HAp scaffold to mimic native bone through a multipass extraction process with the addition of osteoblast-like cells, with pores of 150 ± 20 μm in diameter and with a pore structure of 50 ± 10 μm which is thin enough for rapid bone resorption. With in vivo tests and in vitro tests, they confirmed that the scaffold used is appropriate for graft without inflammatory reactions and bone formation after 8 weeks of implantation. The scaffold's porosity is a critical parameter enabling medium exchange and nutrient diffusion, which is a key role in cell proliferation. So, the optimization of the scaffold's porosity is important to help cell growth, formation of vascularization, and the diffusion of nutrients [67].

Roohani-Esfahani and their co-workers developed a glass–ceramic scaffold, with dimension size 6x6x6mm, by direct ink writing mimicking cortical bone with 600 μm custom-made nozzle. In the work, they concluded that a scaffold with hexagonal pore shapes (450 μm, 550 μm, 900 μm, and 1200 μm) present the highest compressive strength, compared to the other designs [68].

Abbot and co-workers, in 2016, developed a silk scaffold with osteoblasts to evaluate in vitro culture that stimulated bone differentiation and regeneration. In the end, they concluded that it was evident the mineralization in the scaffold with silk seeded with this type of cells [23].

Tovar and co-workers [49] had developed a cylindrical scaffold with 10.5 mm length, 4.5 mm outside diameter and 2.25 mm inside diameter, 330 μm struts, and around 400 μm pore spacing. They used a 330-μm-diameter extrusion nozzle with a velocity of 8 mm/s. The existence of macrometric and micrometric porosity in the scaffold helped in its degradation, which allowed the biomechanical load to the healing bone. This may explain the rapid development of bone properties in the regenerated tissue that is highly indicative of complete healing when it is complemented with the remodeling of the original bone morphology.

Recently, Barba et al. [19, 69] concluded that the geometric parameters of the scaffold, like curvature, influence bone tissue regeneration. They demonstrated that spongy scaffolds with concave pores attracted a large amount of ectopic bone compared with scaffolds with prismatic geometries.

Through the existing studies, both in vivo and in vitro, one can get an idea of both material and biological components essentials to a proper bone regeneration. In the first phase, it is necessary to understand which scaffold design is the most appropriate and which biomaterials are to combine it with the AM technique.

5. Material component

For bone regeneration, it is necessary to consider that the scaffold must restore the normal biomechanical role of the tissue. **Table 2** shows different types of existing materials and their characteristics and some guidelines about how to obtain an ideal scaffold. However, there are other important features that need to be taken into account related to the different biological and physical signals involved in order to simulate the mechanism of remodeling in a natural environment, but more importantly, the scaffold must have the exact mechanical properties to withstand the loads the original bone held [6, 70]. The biomaterials used in the scaffolds must have a suitable rate of degradation in order to support bone regeneration. This rate of degradation depends on the corrosion resistance of the material used, which is affected by the chemical and physical characteristics of the scaffold [36].

Having into consideration the referred above and combining this information with the natural organization of bone (trabecular and cortical), the best strategy for BTE should pass by the use of collagen type I in the trabecular bone region and Hap in the cortical zone [80, 81]. Despite their advantages and the fact that they are already present in the bone native structure, their proper manipulation is only possible through their combination with synthetic polymers. The most suitable are poly(ε-caprolactone) (PCL) or polylactic acid (PLA) because they are both approved by the Food and Drug Administration. PCL is a stable, biocompatible, biodegradable polymer (from 12 to 48 months) and easy to handle to achieve the desired mechanical properties. Due to its low melting point (60°C) [82, 83], it can be easily combined with the collagen. PLA is a biocompatible polymer, more hydrophilic than PCL, and its handling is similar to the use of PCL. Hydrophilicity accelerates polymer degradation as it accelerates polymer and scaffold moisture [84]. However, it has a high melting point, which could be combined with Hap. With these materials, scaffolds can be produced with two methods: conventional and additive manufacturing (AM).

Scaffolds		**References**
Natural polymers	Biomaterials are widely used because of their biocompatibility, degradation, bioactivity, mechanical kinetics, tissue nonspecificity, and their intrinsic structural similarity to the extracellular matrix of native tissues. They also promote biological recognition, which can positively support cell adhesion and function	[71, 72]
Synthetic polymers	Easy to manipulate the properties of the material to achieve the appropriate mechanical behavior. At a microscale it presents the architecture, 3D composition, and active molecular reactive groups. In a macroscale, they have porosity, stiffness, and elasticity	[6]
Metals	Are used in long bones to better attach to the bones where there is minimal movement between the implant and the host tissue and provide physiological loading functionality to the implant site	[15, 73]
Ceramics	Have been used because of their ability to sustain compressive loads	[6, 74, 75]
Ideal scaffolds		
Should exhibit the adequate mechanical properties, pore size, and biological activity, serve as cell support, and guarantee new bone formation and thus the use of more than one material (a natural with a synthetic one)		[76–79]

Table 2.
Characteristics of the different materials used to produce a scaffold.

5.1 Additive manufacturing

Some studies have used conventional methods for producing scaffolds. However, these methods have no adequate control over pore size and design or interconnectivity [8, 85]. In order to address these problems, since the mid-1980s [86], a new manufacturing type of technology called AM has emerged. Its potential is enormous and overcomes the capabilities of the conventional technologies to produce scaffolds with a complex architecture and with the intention to achieve an appropriate mechanical response to the desired application [36].

Nowadays there are several approaches to AM for various applications. The main approaches are fused filament fabrication (FFF), three-dimensional printing (3DP), stereolithography (SLA), and selective laser sintering (SLS). Each process goes through several steps: (i) development of the 3D model through computer-aided design (CAD); (ii) the files are stored in standard triangular language (STL) format, which is a CAD file format that supports 3D printing and computer-aided manufacturing (CAM); and (iii) these files are inserted into the input devices to create 3D models in a layer-by-layer process [36]. In addition, there are still two processes where it uses the same principles of layer manufacturing: selective laser melting (SLM) [87–90] and electron beam melting (EBM) [91–93]. Both are used to produce metal scaffolds, although SLM can also process polymers and ceramics [3, 94, 95].

FFF, **Figure 1**, or melt-extrusion is an extrusion-based process and is the simplest 3D printing method (see **Table 3**) [36, 96]. Fine thermoplastic polymers in the form of filaments or granules are cast and extruded through a nozzle that allows

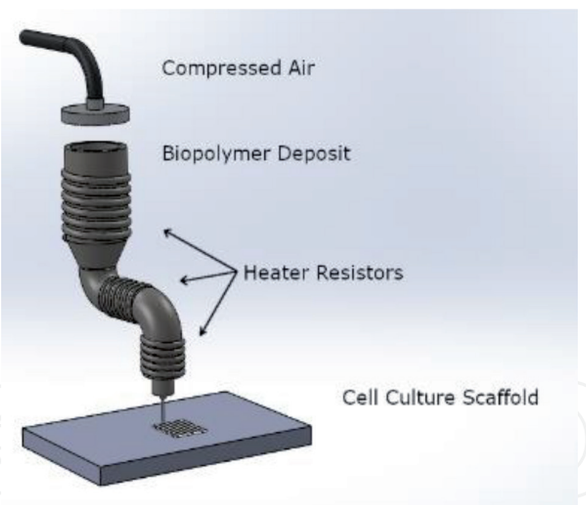

Figure 1.
Fused Filament Fabrication (FFF) process.

Fused filament fabrication	
Advantages	**Disadvantages**
Speed	Poor surface quality
Low cost	Need for heating in the molding process ➔ degradation of polymer materials
Simplicity	
Flexibility	

Table 3.
Advantages and disadvantages of the fused filament fabrication process.

flow in a horizontal and vertical plane (XY plane) [36]. To extrude it is necessary to have heating of the material, which causes degradation. However, the disadvantages of this technique can be overcome. The most suitable and desired mechanical properties can be achieved for the desired purpose with the combination of biomaterials. With this technique, it is already possible to extrude some bioceramics, such as HAp [97–99].

It is critical that the first layer is maintained at a temperature slightly below its set point to ensure successful adhesion between the layers. The 3D structure is determined by several factors, such as nozzle diameter, deposition rate, path spacing of the same layer, layer thickness, and deposition angle [96].

In the FFF technique, it is possible to control layer thickness and print orientation. The structural geometry of scaffolds is determined by the position and orientation of the filaments, which provide various pore shapes such as triangular, parallelogram, hexagonal, and also nonuniform shapes [100]. In this technique, there are two factors that affect the filament size, and consequently the pore size, which are the deposition velocity and the rotational velocity.

5.2 Temporary implants

It is necessary that the scaffolds in bone regeneration be biocompatible, biodegradable, osteoinductive (raising and cell maturation), and osteoconductive (provide a platform for cell growth) [39]. Scaffolds for bone regeneration should meet several specific criteria, such as filling any bone defect, ensuring pore interconnectivity, and having a pore architecture in order to promote bone formation and facilitate the exchange of oxygen bone growth [101–103]. The design of the scaffold can influence both the mechanical properties and cellular behavior [100, 104, 105] as highlighted in **Figure 2**.

A satisfactory bone growth leads to certain requirements. Porosity should be above 50% and pore size between 50 and 400 μm. It is difficult to achieve a "perfect" scaffold for bone regeneration due to pore design and size and a porosity distribution that mimics the native tissue [107, 108]. In the literature, there are no quantitative criteria that specify porosity or pore size or topology for bone regeneration. Porous scaffolds ranging in size from 50 to 500 μm are known to promote cell migration and vascularization, while micropores and nanopores control interaction with proteins and ion exchange with extracellular fluids [19, 109].

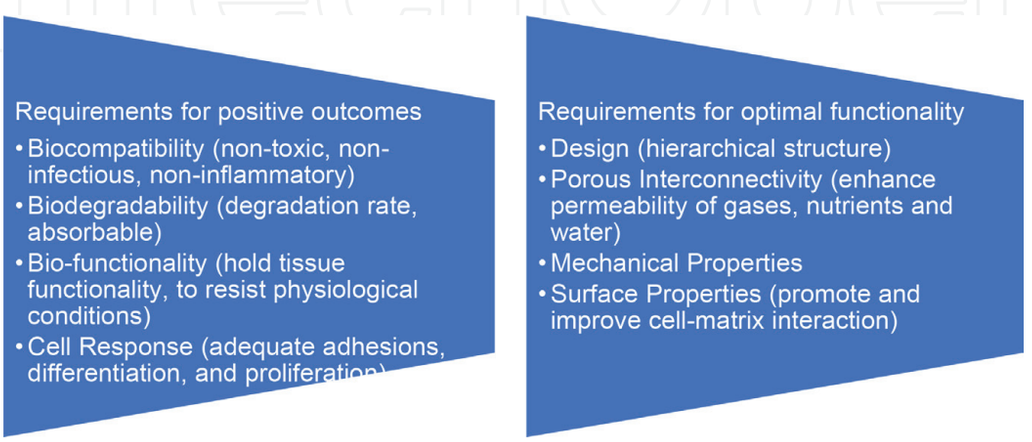

Figure 2.
Scaffold requirements in terms of response (left) and what should be taken into account (right) (adapted from [106]).

6. Biological components

The dogma of molecular biology is the basis for producing most bone cell and ECM components.

Bone remodeling is divided into five stages: activation, resorption, reversal, formation, and, finally, mineralization (see **Figure 3**). It is a process in which the old bone is reabsorbed and there is new bone formation. The cells that are involved in bone remodeling are osteoblasts, osteoclasts, and osteocytes, which actively participate in osseointegration and repair. Osteoclasts activate bone resorption, while osteocytes regulate bone homeostasis and osteoblasts form bone [15, 110].

In addition to bone cells, there are other cell lines that can be used in bone regeneration, which are human embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs), stem cells, and fibroblasts. According to Kuhn [111] and other workers [112, 113], hESCS present a rapidly proliferating rate. However, their transplantation induces uncontrollable spontaneous differentiation and can the teratoma formation may occur. Another type of stem cell is iPSCs. They can differentiate into several cells. However, there are studies that show that these cells can also give rise to teratomas and, in studies that distinguish high-quality lines from the iPSCs, allowed the detection of large duplications of genes that could potentially affect the differentiation and pluripotency of these cells [28, 99]. For these reasons, these cells are not considered the best ones for bone regeneration. Compared to fibroblasts, stem cells have a greater ability to migrate, so these type of cells are the most suitable cells for bone regeneration. Kargozar [58] recently studied the osteogenic potential of different MSCs, such as those derived from human bone marrow, umbilical cord (UC-MSCs) and adipose (AD-MSCs). It concluded that BMMSCs, according to collected histological data, is the most appropriate.

The combination of scaffold, AM, and bioreactor culture shows great potential for creating automated production ecosystems that will enable the formation of commercially available products for BTE application. Efficient nutrient and oxygen transport are important for this type of applications. To this end, bioreactor systems have tried to overcome this difficulty. Rotating-wall vessels are limited to small scaffolds as they do not provide optimal mass transport to the center of the scaffold and are not efficient in osteogenic differentiation, due to shear stress values transmitted to cells.

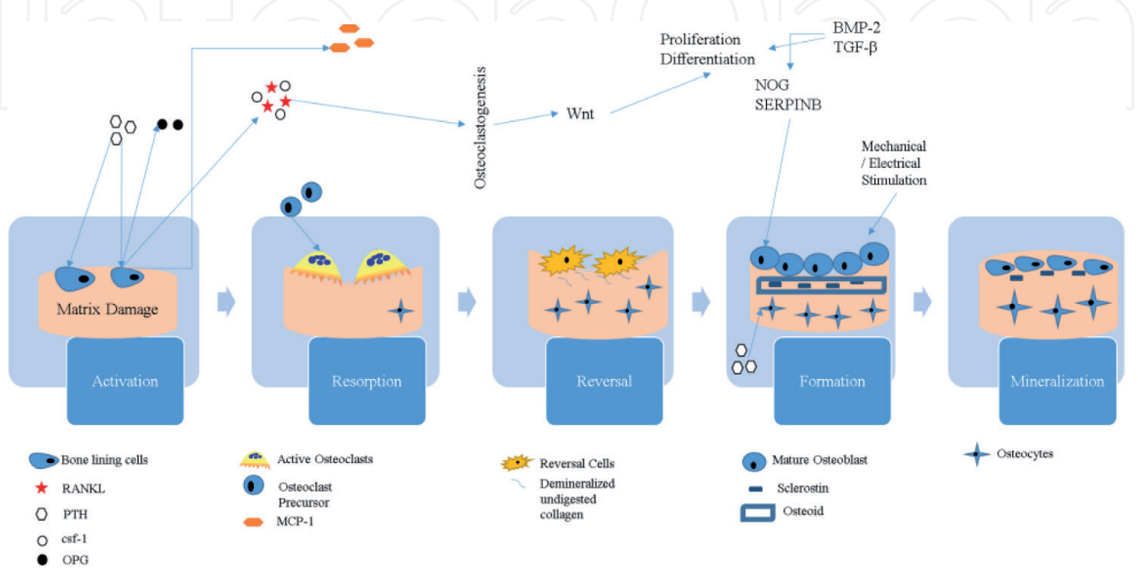

Figure 3.
Bone Remodeling Cycle.

On the other hand, agitated tanks have a major disadvantage regarding the circulating flow pattern that strikes cells against the bioreactor wall, which damages them and can lead to cellular apoptosis [114]. Finally, perfusion bioreactors are the best suited for BTE as they promote oxygenation throughout the whole scaffold, through improved mass transfer and shear stress, can expose cells to mechanical stimulation, and, therefore, obtain a much better cell distribution [6, 39, 61, 115, 116].

7. Optimized approach of a 3D scaffold

Scaffold architectures were designed in SolidWorks 2018 software. The design was bioinspired. This inspiration came from the natural organization of long bones, as represented in **Figure 4**. The diaphysis is composed of cortical bone (external region) which covers the trabecular bone (internal region). The trabecular bone has a larger surface area than the cortical bone and has a bone volume fraction ranging from 5% to a maximum of 60% [117]. It is known that the cortical zone corresponds to ~20% of the total diameter [118]. Bearing this in mind, it is expected that mimicking this type of organization, the mechanical behavior of the final scaffolds would be better and closer to the natural tissue.

The design considered has a height of 10 mm and diameter of 30 mm (see **Figure 5**). Thus, the cortical zone, the outer part of the scaffold, has a thickness of 6 mm and the trabecular zone, the inner part, has 18 mm. In the middle, there is a canal that corresponds to the medullar cavity. As happens in the native tissue, the region corresponding to the trabecular bone presents a higher porosity than the cortical one. So, the proposed scaffold has pores with different sizes between the different parts, bigger in the trabecular and smaller in the cortical

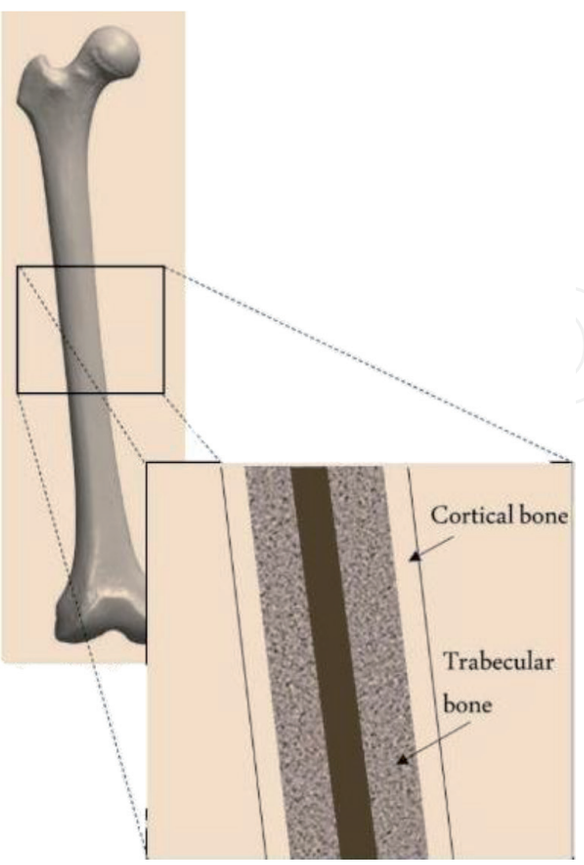

Figure 4.
Natural organization of long bones.

(400 and 300 μm of pore diameters, respectively). According to Zhang et al. [119], these pores are within the required values, since exceeding the pore size of 400 μm, cells do not sense the 3D, resulting in poor ECM production. Moreover, they are organized in a radial way, with a significant difference between the cortical zone and the trabecular zone.

The projected scaffold presents a total porosity of 42%, whereas the cortical part has approximately 5% porosity. This porosity mimics the normal porosity in the native bone (see **Table 1**) as shown by Fernandez-Yague et al. [120] and Wang et al. [3]. The trabecular zone has a porosity of approximately 57%. This porosity is also in agreement with the authors previously mentioned. However, this value is closer to the lower limit. This porosity can be improved by the addition of horizontal channels, but its inclusion would decrease the mechanical behavior of the proposed scaffold.

Since pore interconnectivity is considered by some authors a key point to cell migration and proliferation, another design is proposed, and (see **Figure 6**) it is inspired by a DNA strand. As the scaffold gains height, the base rotates, with a rotation angle of 36°. This was considered to guarantee that the end of the filaments was supported on all layers. Also, in this case, pores diverge gradually, so that the differences between cortical bone and trabecular bone can be noticed.

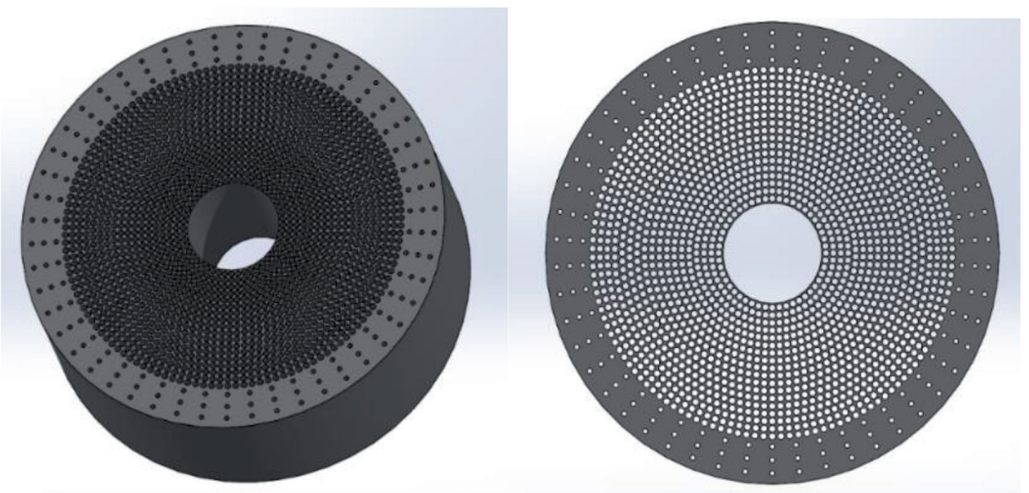

Figure 5.
Cylindrical scaffold.

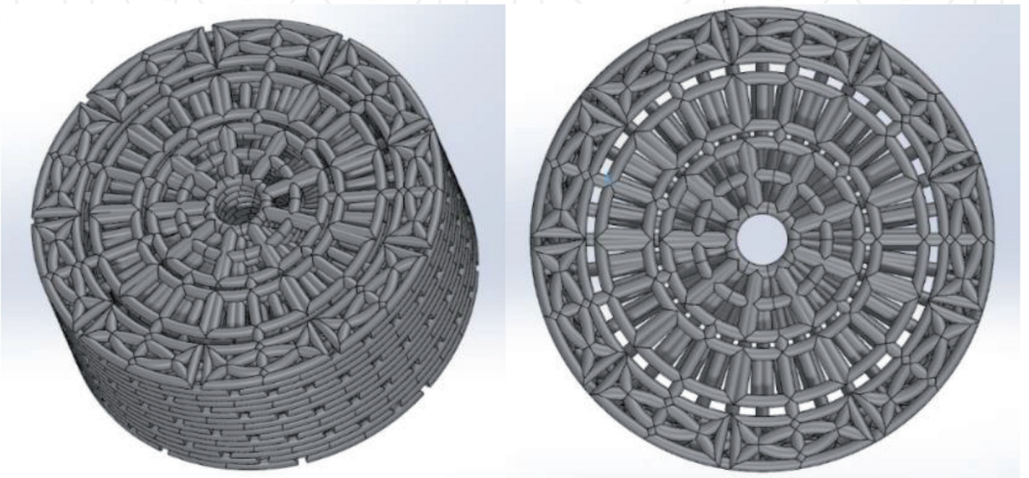

Figure 6.
DNA chain-inspired cylindrical scaffold.

In this scaffold, pores range from 50 to 1500 μm in each layer, which is in agreement with the Zadpoor [121] and Szpalski [39]. However, the minimum required pore size is 100 μm, to make it easier to transport oxygen and nutrients and discard waste products [39, 121, 122]. For proper cell propagation, according to Bael [123], pore size should not pass the 1000 μm. Compared to the previous design presented, this one presents a slightly lower porosity in total, of around 38%, in which the cortical and trabecular parts have 22% and 49% porosity, respectively.

According to the authors Andrzejewska [34], Keaveny et al. [2], and Xiao et al. [124], the porosity of the trabecular zone is near the defined porosity values. However, the porosity of the zone corresponding to the cortical part is far above the maximum value of the defined values found in **Table 1**. In order to decrease this porosity, it is necessary to shrink the pore size used. Despite this limitation, this scaffold already has the advantage of fully interconnected pores, which will facilitate cell growth and the transport of oxygen and nutrients.

The combination of all supports of TE, which were described above, could lead successfully to bone formation. As biomechanics and TE advanced, it is easy to foresee the development of a new model for bone formation in which the use of an original scaffold leads to long bone fracture healing.

8. Conclusions

Bone defects are a constantly growing problem, affecting thousands of people around the world, which causes a loss in life quality, and most of the time, for an active population, it may take long periods of recovery. Until now, there are no synthetic substitutes that meet the mechanical and biological requirements for the long-term cure of critical-size bone defects. To overcome this health problem, the use of temporary biocompatible and biodegradable scaffolds becomes the best choice. Structures produced by AM have superior advantages compared to the conventional techniques, mainly due to better control over the desired architecture. Moreover, the choice of the AM technique to produce these scaffolds is essential to ensure control, namely, in terms of biological, physicochemical, and mechanical properties.

Considering all types of materials available, associated with the desired bone regeneration and the use of synthetic polymers, as PCL or PLA, combined with collagen type I for the trabecular region and Hap for cortical region, seems to be the best strategy to follow. To obtain the designed structures with these biomaterials, the most suitable AM technique is the FFF. For the selection of the final scaffold within the two proposals, further studies need to be performed. However, a third option could be also considered, which would include the cortical region of the first proposed scaffold (ensuring the required mechanical resistance) and the trabecular zone of the second one (assuring a proper porosity and pore interconnectivity to allow cell migration, nutrient, and oxygen exchange).

Among the most commonly used bioreactors for bone regeneration, perfusion bioreactors appear as the most suitable, because it improves osteogenic proliferation and differentiation due to improved mass transfer and adequate shear stress. When making a design proposal for bone regeneration, it is necessary to study the mechanical effects, such as stress and tension, and link them

Acknowledgements

This work is supported by the Fundação para a Ciência e Tecnologia (FCT) and Centro2020 through the Project references UID/Multi/04044/2019,

PAMI—ROTEIRO/0328/2013 (NO. 022158), and MATIS (CENTRO-01-0145-FEDER-000014-3362). It is also funded by the projects insitu.Biomas (POCI-01-0247-FEDER-017771), Bone2Move (PTDC/CVT-CVT/31146/2017), and Stimuli2BioScaffolds (PTDC/EME-SIS/32554/2017).

Conflict of interest

The authors declare no conflict of interest.

Appendices and nomenclature

AM	additive manufacturing
β-TCP	beta-tricalcium phosphate
BMMSCs	bone marrow mesenchymal stem cells
BTE	bone tissue engineering.
CAD	computer-aided design
EBM	electron beam melting
ECM	extracellular matrix
FDA	Food and Drug Administration
FFF	fused filament fabrication
GF	growth factors
Hap	hydroxyapatite
hBMSCs	human bone marrow-derived mesenchymal stem cells
hMSCs	human mesenchymal stem cells
hESCs	human embryonic stem cells
MSC	mesenchymal stem cells
PCL	poly(ε-caprolactone)
PLA	polylactic acid
rBMMSCs	rat bone marrow stromal cell
SLA	stereolithography
SLM	selective laser melting
SLS	selective laser sintering
TCP	tricalcium phosphate
TE	tissue engineering

Author details

Cristiana Fernandes[1], Carla Moura[1], Rita M.T. Ascenso[2], Sandra Amado[1], Nuno Alves[1] and Paula Pascoal-Faria[1*]

1 Centre for Rapid and Sustainable Product Development of the Polytechnic Institute of Leiria, Leiria, Portugal

2 Computer Science and Communication Research Centre, Polytechnic Institute of Leiria, Leiria, Portugal

*Address all correspondence to: paula.faria@ipleiria.pt

References

[1] Gabinete de Estratégia e Planeamento (GEP). Acidentes de Trabalho de 2017. Ministério do Trabalho, Solidariedade e Segurança Social (MTSSS). 2018. p. 228

[2] Keaveny TM, Morgan EF, Niebur GL, Yeh OC. Biomechanics of trabecular bone. Annual Review of Biomedical Engineering. 2001;**3**(1):307-333

[3] Wang X, Xu S, Zhou S, Xu W, Leary M, Choong P, et al. Topological design and additive manufacturing of porous metals for bone scaffolds and orthopaedic implants: A review. Biomaterials. 2016;**83**:127-141. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0142961216000144

[4] Cole JH, van der Meulen MCH. Whole bone mechanics and bone quality. Clinical Orthopaedics and Related Research. 2011;**469**(8): 2139-2149

[5] Shrivats AR, McDermott MC, Hollinger JO. Bone tissue engineering: State of the union. Drug Discovery Today. 2014;**19**(6):781-786. DOI: 10.1016/j.drudis.2014.04.010

[6] Hasan A. Tissue Engineering for Artificial Organs: Regenerative Medicine, Smart Diagnostics and Personalized Medicine. Qatar University. Wiley-VCH Verlag; 2017. Available from: http://onlinelibrary.wiley.com/store/10.1002/9783527689934.ch17/asset/ ch17.pdf?v=1&t=j29txyw2&s=9855d273955de36128e55a5983693ca018f71ea5

[7] De Witte T-M, Fratila-Apachitei LE, Zadpoor AA, Peppas NA. Bone tissue engineering via growth factor delivery: From scaffolds to complex matrices. Regenerative Biomaterials. 2018;**5**(4):197-211. Available from: https://academic.oup.com/rb/article/5/4/197/5035357

[8] Orciani M, Fini M, Di Primio R, Mattioli-Belmonte M. Biofabrication and bone tissue regeneration: Cell source, approaches, and challenges. Frontiers in Bioengineering and Biotechnology. 2017;**5**:1-15. Available from: http://journal.frontiersin.org/article/10.3389/fbioe.2017.00017/full

[9] Jariwala SH, Lewis GS, Bushman ZJ, Adair JH, Donahue HJ. 3D printing of personalized artificial bone scaffolds. 3D Printing and Additive Manufacturing. 2015;**2**(2):56-64. DOI: 10.1089/3dp.2015.0001

[10] Poh PSP, Valainis D, Bhattacharya K, van Griensven M, Dondl P. Optimization of bone scaffold porosity distributions. Scientific Reports. 2019;**9**(1):9170. Available from: http://www.nature.com/articles/s41598-019-44872-2

[11] Schemitsch EH. Size matters: Defining critical in bone defect size! Journal of Orthopaedic Trauma. 2017;**31**(10):S20-S22. Available from: http://insights.ovid.com/crossref?an=00005131-201710005-00005

[12] Oryan A, Alidadi S, Moshiri A, Maffulli N. Bone regenerative medicine: Classic options, novel strategies, and future directions. Journal of Orthopaedic Surgery and Research. 2014;**9**(1):18. Available from: http://josr-online.biomedcentral.com/articles/10.1186/1749-799X-9-18

[13] Leach JK, Mooney DJ. Bone engineering by controlled delivery of osteoinductive molecules and cells. Expert Opinion on Biological Therapy. 2004;**4**(7):1015-1027. DOI: 10.1517/14712598.4.7.1015

[14] Gibbs DMR, Vaezi M, Yang S, Oreffo RO. Hope versus hype: What can additive manufacturing realistically offer trauma and orthopedic surgery?

Regenerative Medicine. 2014;**9**(4):535-549. DOI: 10.2217/rme.14.20

[15] Agarwal R, García AJ. Biomaterial strategies for engineering implants for enhanced osseointegration and bone repair. Advanced Drug Delivery Reviews. 2015;**94**:53-62. DOI: 10.1016/j.addr.2015.03.013

[16] Decoster TA, Gehlert RJ, Mikola EA, Pirela-cruz MA. Management of Posttraumatic Segmental Bone Defects. Journal of the American Academy of Orthopaedic Surgery. 2004;**12**(1):28-38

[17] Garbuz DS, Frcs C, Masri BA, Czitrom AA. Biology of allografting. The Orthopedic Clinics of North America. 1998;**29**(2):199-204

[18] Aghaloo TL, Hadaya D. Basic principles of bioengineering and regeneration. Oral and Maxillofacial Surgery Clinics. 2017;**29**(1):1-7. DOI: 10.1016/j.coms.2016.08.008

[19] Barba A, Maazouz Y, Diez-Escudero A, Rappe K, Espanol M, Montufar EB, et al. Osteogenesis by foamed and 3D-printed nanostructured calcium phosphate scaffolds: Effect of pore architecture. Acta Biomaterialia. 2018;**79**:135-147. DOI: 10.1016/j.actbio.2018.09.003

[20] Manassero M, Viateau V, Deschepper M, Oudina K, Logeart-Avramoglou D, Petite H, et al. Bone regeneration in sheep using acropora coral, a natural resorbable scaffold, and autologous mesenchymal stem cells. Tissue Engineering. Part A. 2013;**19**(13-14):1554-1563

[21] Langer R, Vacanti JP. Tissue engineering. Science (80-). 1993;**260**: 920-926

[22] Loh QL, Choong C. Three-dimensional scaffolds for tissue engineering applications: Role of porosity and pore size. Tissue Engineering. Part B, Reviews. 2013;**19**(6):485-502

[23] Abbott RD, Kimmerling EP, Cairns DM, Kaplan DL. Silk as a biomaterial to support Long-term three-dimensional tissue cultures. ACS Applied Materials & Interfaces. 2016;**8**(34):21861-21868

[24] Abbott RD, Kaplan DL. Strategies for improving the physiological relevance of human engineered tissues. Trends in Biotechnology. 2015;**33**(7):401-407. DOI: 10.1016/j.tibtech.2015.04.003

[25] Giannoudis PV, Harwood PJ, Tosounidis T, Kanakaris NK. Restoration of long bone defects treated with the induced membrane technique: Protocol and outcomes. Injury. 2016;**47**:S53-S61. DOI: 10.1016/S0020-1383(16)30840-3

[26] Gugala Z, Gogolewski S. Regeneration of segmental Diaphyseal defects in sheep tibiae using Resorbable polymeric membranes: A preliminary study. Journal of Orthopaedic Trauma. 1999;**13**(3):187-195

[27] Key JA. The effect of a local calcium depot on osteogenesis and healing of fractures. The Journal of Bone and Joint Surgery. 1934;**16**(1):176-184

[28] Fliefel R, Ehrenfeld M, Otto S. Induced pluripotent stem cells (iPSCs) as a new source of bone in reconstructive surgery: A systematic review and meta-analysis of preclinical studies. Journal of Tissue Engineering and Regenerative Medicine. 2018;**12**(7):1780-1797

[29] Dimitriou R, Jones E, Mcgonagle D, Giannoudis PV. Bone regeneration: Current concepts and future directions. BMC Medicine. 2011;**9**(1):66

[30] Jeon OH, Panicker LM, Lu Q, Chae JJ, Feldman RA, Elisseeff JH. Human

iPSC-derived osteoblasts and osteoclasts together promote bone regeneration in 3D biomaterials. Scientific Reports. 2016;**6**:1-11. DOI: 10.1038/srep26761

[31] Lu H, Liu Y, Guo J, Wu H, Wang J, Wu G. Biomaterials with antibacterial and osteoinductive properties to repair infected bone defects. International Journal of Molecular Sciences. 2016;**17**(3):334

[32] Lu C, Chang Y, Lin S, Li K, Hu Y. Recent progresses in gene delivery-based bone tissue engineering. Biotechnology Advances. 2013;**31**(8):1695-1706. DOI: 10.1016/j.biotechadv.2013.08.015

[33] van Rijt S, Habibovic P. Enhancing regenerative approaches with nanoparticles. Journal of the Royal Society Interface. 2017;**14**(129):20170093

[34] Andrzejewska A. Biomechanical properties of 3D-printed bone models. In: Biosystems. Faculty of Mechanical Engineering, UTP University of Science and Technology in Bydgoszcz. Vol. 176. Poland: Elsevier; 2019. pp. 1-676. DOI: 10.1016/j. biosystems.2019.01.001

[35] Vogl F, Bernet B, Bolognesi D, Taylor WR. Towards assessing cortical bone porosity using low-frequency quantitative acoustics: A phantom-based study. PLoS One. 2017;**12**(9):e0182617. Available from: https://dx.plos.org/10.1371/journal.pone.0182617

[36] Yuan L, Ding S, Wen C. Additive manufacturing technology for porous metal implant applications and triple minimal surface structures: A review. Bioactive Materials. 2019;**4**(1):56-70. DOI: 10.1016/j.bioactmat.2018.12.003

[37] Mills LA, Simpson AHRW. In vivo models of bone repair. Journal of Bone and Joint Surgery. British Volume (London). 2012;**94-B**(7):865-874

[38] Clinton T, Lanyon LE. Regulation of bone formation by applied dynamic loads. The Journal of Bone and Joint Surgery. 1984;**66**:397-402

[39] Szpalski C, Sagebin F, Barbaro M, Warren SM. The influence of environmental factors on bone tissue engineering. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2013;**101**(4):663-675

[40] Cowin SC. Bone poroelasticity. Journal of Biomechanics. 1999;**32**(3):217-238

[41] Peric M, Dumic-Cule I, Grcevic D, Matijasic M, Verbanac D, Paul R, et al. The rational use of animal models in the evaluation of novel bone regenerative therapies. Bone. 2015;**70**:73-86. DOI: 10.1016/j.bone.2014.07.010

[42] Ho-Shui-Ling A, Bolander J, Rustom LE, Johnson AW, Luyten FP, Picart C. Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives. Biomaterials. 2018;**180**:143-162. DOI: 10.1016/j.biomaterials.2018.07.017

[43] Lammens J, Maréchal M, Geris L, Van der Aa J, Van Hauwermeiren H, Luyten FP, et al. Warning about the use of critical-size defects for the translational study of bone repair: Analysis of a sheep Tibial model. Tissue Engineering. Part C, Methods. 2017;**23**(11):694-699

[44] Nather A, David V, Teng JWH, Lee CW, Pereira BP. Effect of autologous mesenchymal stem cells on biological healing of allografts in critical-sized tibial defects simulated in adult rabbits. Annals of the Academy of Medicine, Singapore. 2010;**39**(8):599

[45] Khojasteh A, Eslaminejad MB, Nazarian H, Morad G, Dashti SG, Behnia H, et al. Vertical bone augmentation with simultaneous implant placement using particulate

mineralized bone and mesenchymal stem cells: A preliminary study in rabbit. The Journal of Oral Implantology. 2013;**39**(1):3-13

[46] Lee JY, Choi MH, Shin EY, Kang YK. Autologous mesenchymal stem cells loaded in Gelfoam® for structural bone allograft healing in rabbits. Cell and Tissue Banking. 2011;**12**(4):299-309

[47] Jang DW, Franco RA, Sarkar SK, Lee BT. Fabrication of porous hydroxyapatite scaffolds as artificial bone preform and its biocompatibility evaluation. ASAIO Journal. 2014;**60**(2):216-223

[48] Chowdhary R, Halldin A, Jimbo R, Wennerberg A. Influence of micro threads alteration on osseointegration and primary stability of implants: An FEA and In vivo analysis in rabbits. Clinical Implant Dentistry and Related Research. 2015;**17**(3):562-569

[49] Tovar N, Witek L, Atria P, Sobieraj M, Bowers M, Lopez CD, et al. Form and functional repair of long bone using 3D-printed bioactive scaffolds. Journal of Tissue Engineering and Regenerative Medicine. 2018;**12**(9):1986-1999

[50] Saravanan S, Chawla A, Vairamani M, Sastry TP, Subramanian KS, Selvamurugan N. Scaffolds containing chitosan, gelatin and graphene oxide for bone tissue regeneration in vitro and in vivo. International Journal of Biological Macromolecules. 2017;**104**:1975-1985. DOI: 10.1016/j.ijbiomac.2017.01.034

[51] Smith JO, Tayton ER, Khan F, Aarvold A, Cook RB, Goodship A, et al. Large animal in vivo evaluation of a binary blend polymer scaffold for skeletal tissue-engineering strategies; translational issues. Journal of Tissue Engineering and Regenerative Medicine. 2014;**11**(4):1065-1076

[52] Bragdon CR, Burke D, Lowenstein JD, Connor DOO, Ramamurti B, Jasty M, et al. Differences in stiffness between a cementless and cancellous bone into varying amounts of the interface porous implant vivo in dogs due implant motion. Clinical Orthopaedics. 1996;**11**(8):945-951

[53] Ko J-Y, Park S, Im G-I. Osteogenesis from human induced pluripotent stem cells: An in vitro and in vivo comparison with mesenchymal stem cells. Stem Cells and Development. 2014;**23**(15):1788-1797

[54] Kim HJ, Park J-S. Usage of human mesenchymal stem cells in cell-based therapy: Advantages and disadvantages. Development and Reproduction. 2017;**21**(1):1-10. Available from: http://www.ksdb.org/archive/view_article?pid=dr-21-1-1

[55] Omole AE, Fakoya AOJ. Ten years of progress and promise of induced pluripotent stem cells: Historical origins, characteristics, mechanisms, limitations, and potential applications. PeerJ. 2018;**6**:e4370. Available from: https://peerj.com/articles/4370

[56] Zhang Z. Bone regeneration by stem cell and tissue engineering in oral and maxillofacial region. Frontiers of Medicine in China. 2011;**5**(4):401-413

[57] Hoch AI, Leach JK. Concise review: Optimizing expansion of bone marrow mesenchymal stem/stromal cells for clinical applications. Stem Cells Translational Medicine. 2014;**3**(5):643-652

[58] Kargozar S, Mozafari M, Hashemian SJ, Brouki Milan P, Hamzehlou S, Soleimani M, et al. Osteogenic potential of stem cells-seeded bioactive nanocomposite scaffolds: A comparative study between human mesenchymal stem cells derived from bone, umbilical cord Wharton's jelly, and adipose

tissue. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2018;**106**(1):61-72. DOI: 10.1002/jbm.b.33814

[59] Lv Q, Nair L, Laurencin CT. Fabrication, characterization, and in vitro evaluation of poly(lactic acid glycolic acid)/nano-hydroxyapatite composite microsphere-based scaffolds for bone tissue engineering in rotating bioreactors. Journal of Biomedical Materials Research Part A. 2009;**91**(3):679-691

[60] Hosseinpour S, Ghazizadeh Ahsaie M, Rezai Rad M, Baghani MT, Motamedian SR, Khojasteh A. Application of selected scaffolds for bone tissue engineering: A systematic review. Oral and Maxillofacial Surgery. 2017;**21**(2):109-129

[61] Kleinhans C, Mohan RR, Vacun G, Schwarz T, Haller B, Sun Y, et al. A perfusion bioreactor system efficiently generates cell-loaded bone substitute materials for addressing critical size bone defects. Biotechnology Journal. 2015;**10**(11):1727-1738. DOI: 10.1002/biot.201400813

[62] Kim HJ, Kim UJ, Vunjak-Novakovic G, Min BH, Kaplan DL. Influence of macroporous protein scaffolds on bone tissue engineering from bone marrow stem cells. Biomaterials. 2005;**26**(21):4442-4452

[63] Robling AG, Castillo AB, Turner CH. Biomechanical and molecular regulation of bone remodeling. Annual Review of Biomedical Engineering. 2006;**8**:455-498

[64] Freshney IAN, Estate G, Freshney I. Application of cell cultures to toxicology. In: In Cell Culture Methods for In Vitro Toxicology. Dordrecht: Springer; 2001. pp. 9-26

[65] Meyer U, Wiesmann HP, Kruse-Lösler B, Handschel J, Stratmann U, Joos U. Strain-related bone remodeling in distraction osteogenesis of the mandible. Plastic and Reconstructive Surgery. 1999;**103**(3):800-807. Available from: https://insights.ovid.com/crossref?an=00006534-199903000-00005

[66] Univer- S. Fabrication of Porous Hydroxyapatite Scaffolds as Artificial Bone Preform and its Biocompatibility Evaluation. 2014. pp. 216-223

[67] Lu JX, Flautre B, Anselme K, Hardouin P, Gallur A, Descamps M, et al. Role of interconnections in porous bioceramics on bone recolonization in vitro and in vivo. Journal of Materials Science. Materials in Medicine. 1999;**10**(2):111-120

[68] Roohani-Esfahani SI, Newman P, Zreiqat H. Design and fabrication of 3D printed scaffolds with a mechanical strength comparable to cortical bone to repair large bone defects. Scientific Reports. 2016;**6**(February 2015):1-8. DOI: 10.1038/srep19468

[69] Barba A, Diez-Escudero A, Maazouz Y, Rappe K, Espanol M, Montufar EB, et al. Osteoinduction by foamed and 3D-printed calcium phosphate scaffolds: Effect of nanostructure and pore architecture. ACS Applied Materials & Interfaces. 2017;**9**(48):41722-41736

[70] Frohlich M, Grayson W, Wan L, Marolt D, Drobnic M, Vunjak-Novakovic G. Tissue engineered bone grafts: Biological requirements, tissue culture and clinical relevance. Current Stem Cell Research and Therapy. 2008;**3**(4):254-264. Available from: http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1574-888X&volume=3&issue=4&spage=254

[71] Chen F-M, Liu X. Advancing biomaterials of human origin for tissue engineering. Progress in Polymer

Science. 2016;**53**:86-168. DOI: 10.1016/j.progpolymsci.2015.02.004

[72] Renth AN, Detamore MS. Leveraging "raw materials" as building blocks and bioactive signals in regenerative medicine. Tissue Engineering. Part B, Reviews. 2012;**18**(5):341-362. DOI: 10.1089/ten.teb.2012.0080

[73] Ramazanoglu M, Oshida Y. Osseointegration and bioscience of implant surfaces-current concepts at bone-implant interface. In: Turkyilmaz I, editor. Implant Dentistry-A Rapidly Evolving Practice. Rijeka: InTechOpen; 2011

[74] De Peppo GM, Marolt D. Modulating the biochemical and biophysical culture environment to enhance osteogenic differentiation and maturation of human pluripotent stem cell-derived mesenchymal progenitors. Stem Cell Research & Therapy. 2013;**4**(5):1-11

[75] Connecting Mechanics HR. Bone cell activities in the bone remodeling process: An integrated finite element modeling. Frontiers in Bioengineering and Biotechnology. 2014;**2**(April): 1-12. Available from: http://journal.frontiersin.org/article/10.3389/fbioe.2014.00006/abstract

[76] Chesnutt BM, Viano AM, Yuan Y, Yang Y, Guda T, Appleford MR, et al. Design and characterization of a novel chitosan/nanocrystalline calcium phosphate composite scaffold for bone regeneration. Journal of Biomedical Materials Research Part A. 2009;**88**(2):491-502

[77] Hennessy KM, Pollot BE, Clem WC, Phipps MC, Sawyer AA, Culpepper BK, et al. The effect of collagen I mimetic peptides on mesenchymal stem cell adhesion and differentiation, and on bone formation at hydroxyapatite surfaces. Biomaterials. 2009;**30**(10):1898-1909. DOI: 10.1016/j.biomaterials.2008.12.053

[78] Cao H, Kuboyama N. A biodegradable porous composite scaffold of PGA/β-TCP for bone tissue engineering. Bone. 2010;**46**(2):386-395. DOI: 10.1016/j.bone.2009.09.031

[79] Fu SZ, Ni PY, Wang BY, Chu BY, Zheng L, Luo F, et al. Injectable and thermo-sensitive PEG-PCL-PEG copolymer/collagen/n-HA hydrogel composite for guided bone regeneration. Biomaterials. 2012;**33**(19):4801-4809. DOI: 10.1016/j.biomaterials.2012.03.040

[80] Murphy CM, Schindeler A, Gleeson JP, Yu NYC, Cantrill LC, Mikulec K, et al. A collagen–hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates. Acta Biomaterialia. 2014;**10**(5):2250-2258. DOI: 10.1016/j.actbio.2014.01.016

[81] Wahl D, Czernuszka J. Collagen-hydroxyapatite composites for hard tissue repair. European Cells and Materials. 2006;**11**:43-56

[82] Pereira RF, Freitas D, Tojeira A, Almeida HA, Alves N, Bártolo PJ. Computer modelling and simulation of a bioreactor for tissue engineering. International Journal of Computer Integrated Manufacturing. 2014;**27**(10):946-959. DOI: 10.1080/0951192X.2013.812244

[83] Dorati R, DeTrizio A, Modena T, Conti B, Benazzo F, Gastaldi G, et al. Biodegradable scaffolds for bone regeneration combined with drug-delivery systems in osteomyelitis therapy. Pharmaceuticals. 2017;**10**(4):96. Available from: http://www.mdpi.com/1424-8247/10/4/96

[84] Yao Q, Cosme JGL, Xu T, Miszuk JM, Picciani PHS, Fong H, et al. Three dimensional electrospun

PCL/PLA blend nanofibrous scaffolds with significantly improved stem cells osteogenic differentiation and cranial bone formation. Biomaterials. 2017;**115**:115-127. DOI: 10.1016/j.biomaterials.2016.11.018

[85] Roseti L, Parisi V, Petretta M, Cavallo C, Desando G, Bartolotti I, et al. Scaffolds for bone tissue engineering: State of the art and new perspectives. Materials Science and Engineering: C. 2017;**78**:1246-1262. DOI: 10.1016/j.msec.2017.05.017

[86] Tang D, Tare RS, Yang LY, Williams DF, Ou KL, Oreffo ROC. Biofabrication of bone tissue: Approaches, challenges and translation for bone regeneration. Biomaterials. 2016;**83**:363-382. DOI: 10.1016/j.biomaterials.2016.01.024

[87] Van der Stok J, Van der Jagt OP, Amin Yavari S, De Haas MFP, Waarsing JH, Jahr H, et al. Selective laser melting-produced porous titanium scaffolds regenerate bone in critical size cortical bone defects. Journal of Orthopaedic Research. 2013;**31**(5):792-799. DOI: 10.1002/jor.22293

[88] Sing SL, Wang S, Agarwala S, Wiria FE, Ha TMH, Yeong WY. Fabrication of titanium based biphasic scaffold using selective laser melting and collagen immersion. International Journal of Bioprinting. 2017;**3**(1):1719-1722. Available from: http://www.sciencemag.org/content/299/5613/1719.abstract

[89] Guo Y, Wu J, Xie K, Tan J, Yang Y, Zhao S, et al. Study of bone regeneration and osteointegration effect of a novel selective laser-melted titanium-tantalum-niobium-zirconium alloy scaffold. ACS Biomaterials Science & Engineering. 2019;**5**(12):6463-6473. DOI: 10.1021/acsbiomaterials.9b00909

[90] Zhang B, Pei X, Zhou C, Fan Y, Jiang Q, Ronca A, et al. The biomimetic design and 3D printing of customized mechanical properties porous Ti_6Al_4V scaffold for load-bearing bone reconstruction. Materials and Design. 2018;**152**:30-39. DOI: 10.1016/j.matdes.2018.04.065

[91] Ataee A, Li Y, Fraser D, Song G, Wen C. Anisotropic Ti-6Al-4V gyroid scaffolds manufactured by electron beam melting (EBM) for bone implant applications. Materials and Design. 2018;**137**:345-354. DOI: 10.1016/j.matdes.2017.10.040

[92] Surmeneva MA, Surmenev RA, Chudinova EA, Koptioug A, Tkachev MS, Gorodzha SN, et al. Fabrication of multiple-layered gradient cellular metal scaffold via electron beam melting for segmental bone reconstruction. Materials and Design. 2017;**133**:195-204. DOI: 10.1016/j.matdes.2017.07.059

[93] Lv J, Xiu P, Tan J, Jia Z, Cai H, Liu Z. Enhanced angiogenesis and osteogenesis in critical bone defects by the controlled release of BMP-2 and VEGF: Implantation of electron beam melting-fabricated porous Ti_6Al_4V scaffolds incorporating growth factor-doped fibrin glue. Biomedical Materials [Internet]. 2015;**10**(3):035013. DOI: 10.1088/1748-6041/10/3/035013

[94] Ngo TD, Kashani A, Imbalzano G, Nguyen KTQ, Hui D. Additive manufacturing (3D printing): A review of materials, methods, applications and challenges. Composites Part B: Engineering. 2018;**143**(February):172-196. DOI: 10.1016/j.compositesb.2018.02.012

[95] Zadpoor AA, Malda J. Additive manufacturing of biomaterials, tissues, and organs. Annals of Biomedical Engineering. 2017;**45**(1):1-11. Available from: http://link.springer.com/10.1007/s10439-016-1719-y

[96] Ji K, Wang Y, Wei Q, Zhang K, Jiang A, Rao Y, et al. Application of

3D printing technology in bone tissue engineering. Bio-Design and Manufacturing. 2018;**1**(3):203-210. Available from: http://link.springer.com/10.1007/s42242-018-0021-2

[97] Rodriguez G, Dias J, D'Ávila MA, Bártolo P. Influence of hydroxyapatite on extruded 3D scaffolds. Procedia Engineering. 2013;**59**(November 2015):263-269. DOI: 10.1016/j.proeng.2013.05.120

[98] 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. 2016:1-10. Available from: https://www.hindawi.com/journals/bmri/2016/1596157/

[99] D'Amora U, Russo T, Gloria A, Rivieccio V, D'Antò V, Negri G, et al. 3D additive-manufactured nanocomposite magnetic scaffolds: Effect of the application mode of a time-dependent magnetic field on hMSCs behavior. Bioactive Materials. 2017;**2**(3):138-145

[100] Gleadall A, Visscher D, Yang J, Thomas D, Segal J. Review of additive manufactured tissue engineering scaffolds: Relationship between geometry and performance. Burns and Trauma. 2018;**6**(1):19. Available from: https://burnstrauma.biomedcentral.com/articles/10.1186/s41038-018-0121-4

[101] Hollister SJ, Murphy WL. Scaffold translation: Barriers between concept and clinic. Tissue Engineering. Part B, Reviews [Internet]. 2011;**17**(6):459-474. DOI: 10.1089/ten.teb.2011.0251

[102] Billström GH, Blom AW, Larsson S, Beswick AD. Application of scaffolds for bone regeneration strategies: Current trends and future directions. Injury [Internet]. 2013;**44**:S28-S33. DOI: 10.1016/S0020-1383(13)70007-X

[103] Wu S, Liu X, Yeung KWK, Liu C, Yang X. Biomimetic porous scaffolds for bone tissue engineering. Materials Science & Engineering R: Reports. 2014;**80**:1-36. DOI: 10.1016/j.mser.2014.04.001

[104] An J, Teoh JEM, Suntornnond R, Chua CK. Design and 3D printing of scaffolds and tissues. Engineering [Internet]. 2015;**1**(2):261-268. DOI: 10.15302/J-ENG-2015061

[105] Hollister S. Porous scaffold design for tissue engineering. Nature Materials. 2005;4(7):518. Available from: http://sfx.lib.umich.edu:9003/sfx_local?url_ver=Z39.88-2004&url_ctx_fmt=info:ofi/fmt:kev:mtx:ctx&rft_val_fmt=info:ofi/fmt:kev:mtx:journal&rft.atitle=Porous scaffold design for tissue engineering (vol 4, pg 518, 2005)&rft.auinit=S&rft.aulast=Hollister&rft

[106] Karioja H, Jantunent P. Infotech Oulu Annual Report 2016—Electronics Materials, Packaging and Reliability Techniques (EMPART). University Oulu; 2017. Available from: https://www.oulu.fi/infotech/annual_report/2016/empart [cited 21 June 2019]

[107] Arabnejad S, Johnston RB, Ann J, Singh B, Tanzer M, Pasini D. High-strength porous biomaterials for bone replacement: A strategy to assess the interplay between cell morphology, mechanical properties, bone ingrowth and manufacturing constraints. Acta Biomaterialia. 2016;**30**:345-356. DOI: 10.1016/j.actbio.2015.10.048

[108] Heinl P, Müller L, Körner C, Singer RF, Müller FA. Cellular Ti-6Al-4V structures with interconnected macro porosity for bone implants fabricated by selective electron beam melting. Acta Biomaterialia. 2008;**4**(5):1536-1544

[109] Hannink G, Arts JJC. Bioresorbability, porosity and mechanical strength of bone substitutes:

What is optimal for bone regeneration? Injury. 2011;**42**(Suppl. 2):S22-S25. DOI: 10.1016/j.injury.2011.06.008

[110] Dean DD, Oefinger J, Schwartz Z, Boyan BD, Lohmann CH, Bonewald LF. Implant surface characteristics modulate differentiation behavior of cells in the osteoblastic lineage. Advances in Dental Research. 2009;**13**(1):38-48

[111] Kuhn LT, Liu Y, Boyd NL, Dennis JE, Jiang X, Xin X, et al. Developmental-like bone regeneration by human embryonic stem cell-derived mesenchymal cells. Tissue Engineering. Part A. 2014;**20**(1-2):365-377. DOI: 10.1089/ten.tea.2013.0321

[112] Barberi T, Willis LM, Socci ND, Studer L. Derivation of multipotent mesenchymal precursors from human embryonic stem cells. PLoS Medicine. 2005;**2**(6):e161. Available from: https://dx.plos.org/10.1371/journal.pmed.0020161

[113] Trounson A. Human embryonic stem cells: Mother of all cell and tissue types. Reproductive Biomedicine Online. 2002;**4**(December):58-63. DOI: 10.1016/S1472-6483(12)60013-3

[114] Sladkova M, de Peppo G. Bioreactor systems for human bone tissue engineering. Processes. 2014;**2**(2):494-525. Available from: http://www.mdpi.com/2227-9717/2/2/494/

[115] Kim J, Kennedy K, Vunjak-Novakovic G. Bioreactors in regenerative medicine. In: Principles of Regenerative Medicine. Columbia University. New York, NY, United States: Elsevier; 2019. pp. 787-803. Available from: https://linkinghub.elsevier.com/retrieve/pii/B978012809880600045X

[116] Asadi-eydivand M, Solati-hashjin M, Farzad A, Osman NAA. Effect of technical parameters on porous structure and strength of 3D printed calcium sulfate prototypes. Robotics and Computer-Integrated Manufacturing. 2016;**37**:57-67. DOI: 10.1016/j.rcim.2015.06.005

[117] Drača N, Tikvica A, Vukičević S, Eljuga D, Semenski D, Brnčić M. Biomechanical properties of bones from rats treated with sevelamer. Collegium Antropologicum. 2011;**35**(2):557-563

[118] Long Y, Leslie WD, Luo Y. Study of DXA-derived lateral–medial cortical bone thickness in assessing hip fracture risk. Bone Reports. 2015;**2**:44-51. DOI: 10.1016/j.bonr.2015.02.003

[119] Zhang L, Yang G, Johnson BN, Jia X. Three-dimensional (3D) printed scaffold and material selection for bone repair. Acta Biomaterialia. 2019;**84**:16-33. DOI: 10.1016/j.actbio.2018.11.039

[120] Fernandez-Yague MA, Abbah SA, McNamara L, Zeugolis DI, Pandit A, Biggs MJ. Biomimetic approaches in bone tissue engineering: Integrating biological and physicomechanical strategies. Advanced Drug Delivery Reviews. 2015;**84**:1-29. DOI: 10.1016/j.addr.2014.09.005

[121] Zadpoor AA. Bone tissue regeneration: The role of scaffold geometry. Biomaterials Science 2015;3(2):231-245. Available from: http://xlink.rsc.org/?DOI=C4BM00291A

[122] Takahashi Y, Tabata Y. Effect of the fiber diameter and porosity of non-woven PET fabrics on the osteogenic differentiation of mesenchymal stem cells. Journal of Biomaterials Science. Polymer Edition. 2004;**15**(1):41-57. DOI: 10.1163/156856204322752228

[123] 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 Ti_6Al_4V bone scaffolds. Acta Biomaterialia. 2012;**8**(7):2824-2834. DOI: 10.1016/j.actbio.2012.04.001

[124] Xiao W, Wang Y, Pacios S, Li S, Graves DT. Cellular and molecular aspects of bone remodeling. In: Kantarci A, Will L, Yen S, editors. Tooth Movement Frontiers of Oral Biology. 2016. pp. 9-16. Available from: https://www.karger.com/Article/FullText/351895