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MAGNETIC SCAFFOLDS FOR BONE REGENERATION

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Abstract

Tissue engineering focuses on developing biological substitutes with the aim of restoring, maintaining or improving tissue function. Bone repair and regeneration call for the use of biodegradable scaffolds, which are synthetic bone grafts whose function is to provide a temporary template for bone formation. Numerous biomaterials have been studied to compose scaffolds, being ceramics and polymers the most used. Recently, research has begun incorporating magnetism in bone regeneration, showing that bone cells demonstrate a positive response to this type of stimuli and that bone development is enhanced by it. Thus, the present work reviews the biomaterials that are used in bone tissue engineering, as well as the requirements that scaffolds must meet, and the techniques employed to produce them. Additionally, the aim of this thesis was to find the achievements of the studies in this field regarding magnetic scaffolds.

Key Words

Bioceramics; Bone Tissue Engineering; Magnetic Fields; Magnetic Nanoparticles; Magnetic Scaffolds; Polymers.

Resumo

A área de engenharia de tecidos foca-se no desenvolvimento de substitutos biológicos com o objetivo de restaurar, manter ou melhorar a função de tecidos. A reparação e regeneração óssea requerem a utilização de *scaffolds* biodegradáveis, que são enxertos ósseos sintéticos cuja função é servirem de suporte temporário para a formação óssea. Para esse efeito, têm vindo a ser estudados inúmeros biomateriais para desenvolver *scaffolds*, sendo os cerâmicos e polímeros os mais aplicados. Recentemente, a investigação nesta área começou também ela a focar-se no efeito do magnetismo na regeneração óssea, demonstrando que as células ósseas reagem positivamente a este tipo de estímulo e que a formação óssea é potenciada pelo mesmo. Desta forma, no presente trabalho foram revistos os biomateriais que são utilizados na área de engenharia de tecidos ósseos, bem como os requisitos que os *scaffolds* devem cumprir, e as técnicas utilizadas para os produzir. Além disso, esta tese teve como objetivo primordial explorar e compreender os trabalhos que já foram desenvolvidos no âmbito do desenvolvimento de *scaffolds* magnéticos para regeneração óssea.

Palavras-Chave

Biocerâmicos; Campos Magnéticos; Engenharia de Tecidos Ósseos; Nanopartículas Magnéticas; Polímeros; Scaffolds Magnéticos.

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To all my friends, for all the unforgettable moments and for the happiness they bring into my life.

Lastly, to my parents, sister and grandfather, for the unconditional love and support in every moment, and for building me as who I am.

O que impede de saber não são nem o tempo nem a inteligência,
mas somente a falta de curiosidade.

Agostinho da Silva

Contents

Figure List

Table List

Abbreviations

3D	three-dimensional
ALP	alkaline phosphatase
AMF	alternating magnetic field
BCP	biphasic calcium phosphates
BMP	bone morphological protein
CAD	computer aided design
ECM	extracellular matrix
EMF	electromagnetic field
FDA	United States Food and Drug Administration
GA	glycolic acid
HA	hydroxyapatite
IONP	iron oxide nanoparticle
LA	lactic acid
MGDT	magnetically guided drug targeting
MNP	magnetic nanoparticle
MRI	magnetic resonance imaging
PEG	polyethylene glycol
PCL	polycaprolactone
PDLA	poly(D-lactic) acid
PDLLA	Poly-D,L-lactic acid
PEMF	pulsed electromagnetic field
PGA	polyglycolic acid
PLA	polylactic acid
PLGA	poly(lactic-co-glycolic acid)
PLLA	poly(L-lactic acid)
RMF	rotating magnetic field
SMF	static magnetic field
SPION	superparamagnetic iron oxide nanoparticle
TCP	tricalcium phosphate
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

Introduction

Bone tissue engineering arose with the necessity to fix bone injuries that bone's natural process of remodelling cannot repair. These injuries can be the result of, for example, fractures or degenerative diseases, as is the case of osteoporosis, a bone disease characterized by structural deterioration of bone tissue and low bone mass. In 2018, osteoporosis affected around 200 million women worldwide. This is an expanding issue given the rise of the elderly population, which is more likely to suffer from bone diseases. In fact, bone is the second most transplanted tissue worldwide, after blood [1, 2].

Autografts and allografts are widely used for this purpose, although they have several limitations, including risks of disease transmission, rejection and improper fixation. Furthermore, there is limited supply. For these reasons, studies in the field of bone tissue engineering focus on developing effective synthetic bone grafts that can be inserted in the bone to promote its regeneration. Figure 1 contains statistics of the market of bone grafts and substitutes that were analysed from 2014 to 2018, and the projection it is made for the following years until 2026, where it is possible to foresee a growing application of synthetic bone grafts [1, 3].

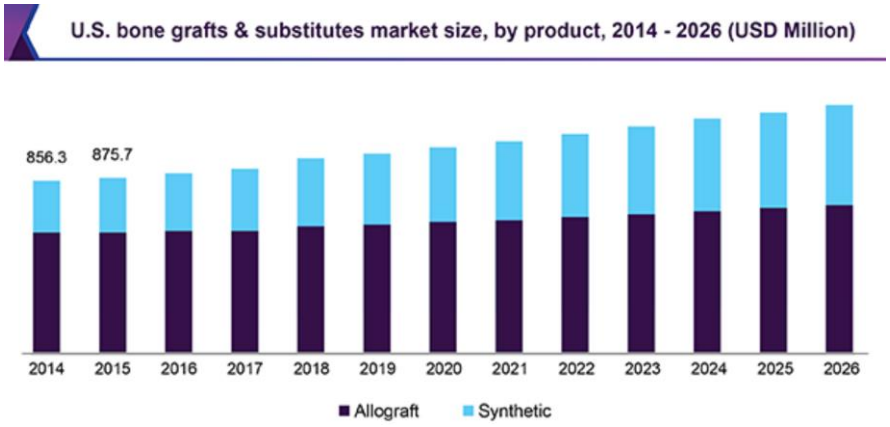

Figure 1: The market size of bone grafts and substitutes in the U.S.A. between 2014 and 2026 [3]

Scaffolds are synthetic bone grafts that work as temporary matrices that biodegrade after the regeneration and proliferation of bone cells. Several biomaterials can be applied in scaffolds, each with its advantages and limitations. Ceramics and polymers are the most studied materials for bone tissue engineering, and research in the field has tested the behaviour of scaffolds produced with several types of these materials both *in vitro* and *in vivo*. Polymer/ceramic composites have also been studied. However, although composite scaffolds exhibit better mechanical properties and biological

behaviour than scaffolds produced from a single type of material, osteoinduction can still be improved.

Bone is a tissue that continuously remodels itself as a result of mechanical loading. The normal locomotion of the body creates stress on bones, thus contributing to the maintenance of bone mineral density and strength. In the same way that bone reacts to external mechanical stimuli, it has been suggested that it may also respond to magnetic stimuli. In this regard, magnetism has been incorporated in bone regeneration studies, with the aim of analysing the behaviour of bone cells facing such stimulus. Magnetic scaffolds have been reported to promote osteoblast proliferation and differentiation, leading to further studies related to this subject in order to validate and find explanations for the observed previous results.

Chapter 1: Bone

Bone is a mineralized hard tissue that provides support to the body and protects vital organs like those in the cranial and thoracic cavities. It is also essential to store calcium, phosphate and other ions that can be released in a controlled manner to balance concentrations in body fluids [4, 5].

1.1. Architectural Structure of Bone

Bone tissue consists of hierarchical structures that go from macroscale to sub-nanoscale, as represented in figure 2. At the macroscopic scale, bone is divided into cortical and trabecular structures. The human body is composed of 80% cortical bone and 20% trabecular bone. These values represent the overall ratio, since cortical/trabecular ratio differs from one part of the body to another. Cortical bone is also referred to as compact bone and it is the portion that surrounds the marrow space. Its density and stiffness confer support against muscle contraction and resistance to fractures and crack propagation. Trabecular bone, also called cancellous bone, is much less dense than cortical bone. It exists mainly in long bones and it has a sponge-like structure. The high porosity of trabecular bone makes its surface area about eight times greater than cortical bone's. For this reason, the exchange of calcium and phosphate ions is much faster in this portion of bone rather than in cortical bone, and therefore trabecular bone is metabolically more active and can be remodelled more frequently [6-8].

At microscopic scale (10-500 μm), cortical bone is composed of basic units called osteons, that are cylindrical structures consisting of concentric lamellae. In the centre of each lamella there is a Haversian canal through which vessels and nerves pass. The anisotropic orientation of osteons makes the cortical bone strength vary depending on whether it is under tension, compression or torsion. The basic units of trabecular bone are trabeculae, which are plate and rod-shaped interconnected structures that form a three-dimensional network and confer porosity to bone. At the submicroscopic scale (1-10 μm), it is possible to observe lamellae in both cortical and trabecular bone. These lamellae are formed by nanoscopic (10-1000 nm) and sub-nanoscopic (less than 100 nm) collagen fibres to which minerals are discontinuously attached [9, 10].

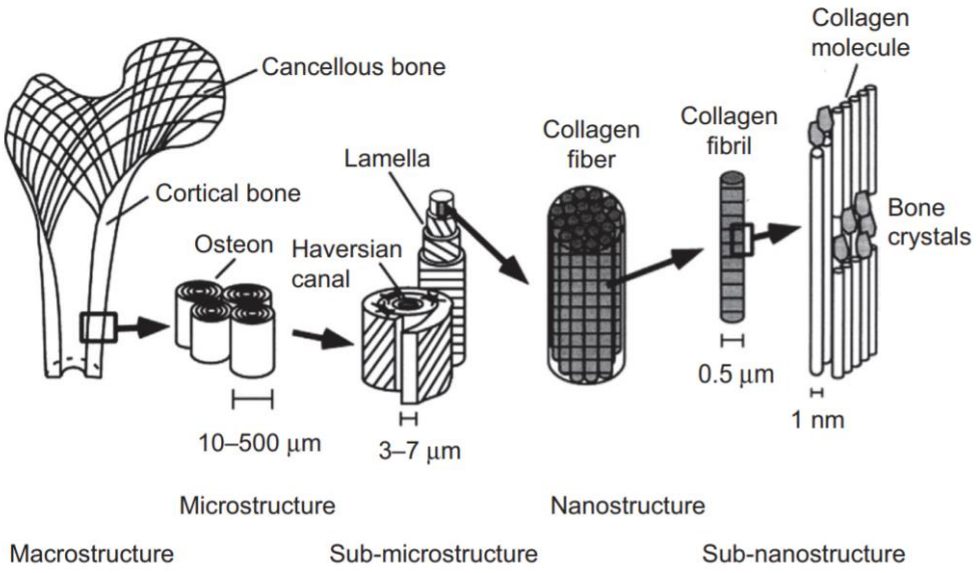

Figure 2: Hierarchical structure of bone in varied length scales [9]

1.2. Chemical Composition of Bone

Bone matrix is a nanostructured composite material consisting of organic (about 20 to 30 wt%) and inorganic (about 65 to 70 wt%) components. The inorganic portion serves as an ion reservoir for mainly phosphorus and calcium, although there are other ions such as sodium and magnesium. Calcium and phosphate ions compose a natural apatite in the shape of crystals that has a chemical composition similar to hydroxyapatite [9, 11].

A collagen-based polymeric matrix makes up the organic portion of bone, which is called the osteoid and is responsible for bounding the ceramic crystals. The osteoid is mainly composed of fibre-shaped type I collagen, a protein whose triple helix structure provides strength in tension due to the crosslinks between helixes. Other constituents of the organic fraction are proteoglycans, whose function is to inhibit mineralization of the bone, and non-collagenous proteins such as osteopontin and osteocalcin. Water is also part of bone matrix, in a total of 5 to 10 wt% of its composition [11].

1.3. Bone Cells

The adaption of the skeleton to mechanical use and fracture healing are aspects that require remodelling of the bone, which consists of renewing the bone tissue in order to maintain bone strength. The process is controlled by the four types of cells: osteoblasts, osteocytes, osteoclasts and lining cells [4, 8].

Osteoblasts derive from multipotent mesenchymal stem cells that exist in bone marrow. Their function is to synthesize the organic components of the bone matrix, as they are responsible for the production of type I collagen and other proteins like osteocalcin and osteonectin. Osteoblasts are also essential for the mineralization of the matrix, since they have the ability to concentrate calcium and phosphate ions in order to form hydroxyapatite crystals. Once they have finished their role in bone forming, they can either convert into lining cells, or end up surrounded by the matrix, becoming osteocytes. Osteoblasts are known to express high quantities of alkaline phosphatase (ALP) anchored to their plasma membrane. This increase of ALP levels in blood is used as an indicator during the formation of bone tissue [5, 8, 12, 13].

Osteocytes are terminally differentiated osteoblasts found in lacunae and work in a network of intercellular communication channels via gap junctions. This results in a syncytium that confers a mechanoreceptor function to this type of cells, allowing them to detect the need for repair of damage and transmit this information in order to obtain a response, thus managing bone remodelling [8, 13].

Lining cells are elongated cells that lie on top of a layer of unmineralized collagen matrix that covers the surface of quiescent bone (bone that is not undergoing remodelling). Since osteoclasts cannot attach to this unmineralized layer, lining cells secrete collagenase, that will remove this layer so that osteocytes can adhere to the bone. Lining cells derive from osteoblasts that have completed their function in bone forming [12].

Osteoclasts are multinucleated cells responsible for removing calcified bone matrix. They derive from hematopoietic cells and are the only cells capable of resorption of the bone tissue. As they degrade the mineralized matrix, osteoclasts produce small pits called resorption bays or Howship's lacunae [5, 8, 12].

1.3.1. Osteoblast Differentiation

Cell differentiation is the process through which a cell changes from one cell type to a more specialized type. Osteoblasts derive from multipotent mesenchymal stem cells that transform into preosteoblasts that later evolve to differentiated bone cells. Osteoblast differentiation can be divided in three stages: cell proliferation, matrix maturation and matrix mineralization, and each of these phases is characterized by the expression of certain substances that can work as indicators. In the case of proliferation, it is possible to detect some matrix proteins such as fibronectin, TGF-β and type I collagen, while matrix maturation corresponds to the maximal expression of alkaline phosphatase. As for matrix mineralization, in the beginning of this phase there

is the expression of genes for proteins like osteocalcin and osteopontin, and when this stage is completed, it is possible to observe calcium deposition [14, 15].

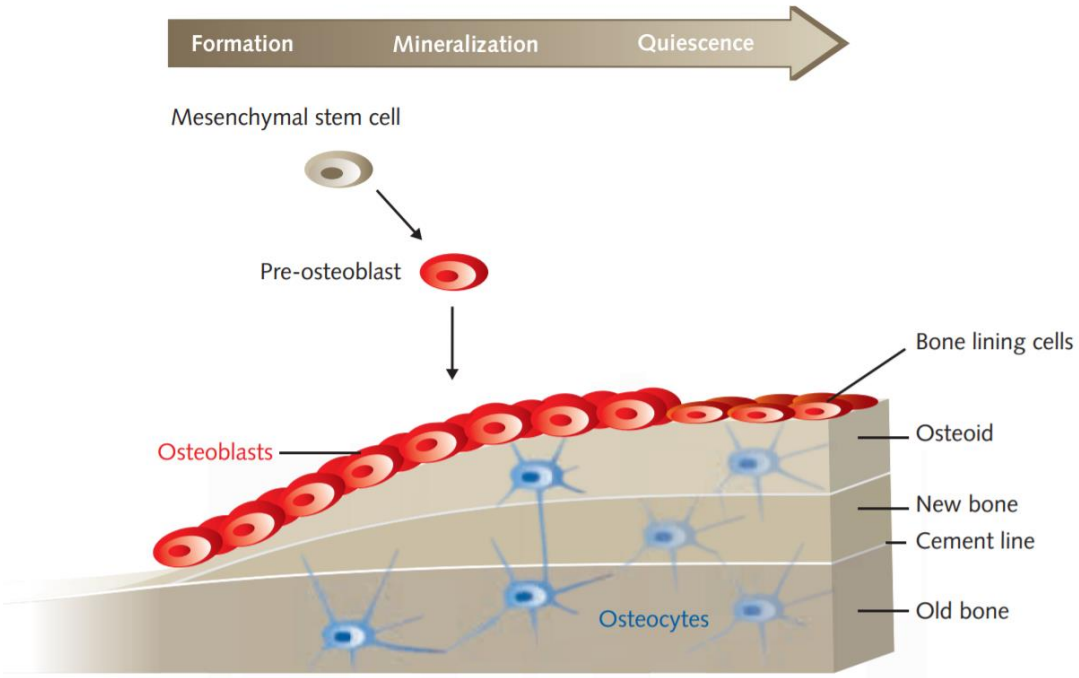

Figure 3: Schematic representation of osteoblast differentiation [14]

1.4. Bone Remodelling

Although it appears as an inert organ, bone is under continuous remodelling, which is the process that assures the renovation and maintenance of bone strength and mineral homeostasis. This process comes as a response to mechanical loading. For that reason, increasing density and strength are found in areas exposed to stress, while unstimulated areas exhibit loss of density and bone mass. It involves the degradation of dysfunctional bone by osteoclasts and biosynthesis of new bone by osteoblasts [16].

The remodelling process is triggered by the death of osteocyte cells. Once osteocyte apoptosis takes place, it leads to osteoblasts activation and increases osteoclast precursors, which originate active osteoclasts. The process then begins with the resorption of bone tissue by the osteoclasts, where the mineral fraction of the matrix is dissolved in an acidic environment that is characteristic of the resorption site. This degradation of the matrix results in Howship's lacunae on the surface of trabecular bone and Haversian canals in cortical bone. The degraded bone matrix components are removed through endocytosis and transcytosis [8, 12, 13].

Afterwards, osteoblasts start producing new collagen matrix and regulating its mineralization by destroying mineralization inhibitors such as pyrophosphate and proteoglycans. This is achieved with the release of matrix vesicles that contain calcium and phosphate and enzymatically destroy such inhibitors. The mineralization of the osteoid matrix is essential for the deposition of hydroxyapatite on the type I collagen strands. Some osteoblasts become entrapped in the matrix and differentiate into osteocytes, that connect to bone surface lining cells through the canalicular network where they are inserted. There, they play their role as mechanosensory cells that detect the need for repair of damage and transmit signals to trigger an appropriate response. New bone is deposited in layers inside the channels that result from the resorption of the matrix, which results in the formation of concentric lamellae. This way, the result of each remodelling cycle is the formation of a new osteon [8, 12, 13].

Bone is a complex tissue that requires deep understanding of its structure and its remodelling process, in order to understand the requirements that bone grafts must meet and the procedure that is necessary to design a synthetic bone graft that will mimic the bone structure, as well as the materials that are adequate for this matter.

Chapter 2: Bone Tissue Engineering

Tissue engineering arose from the necessity to repair tissues and organs that the body cannot self-heal. Regarding bone, it is the case of defects that are greater than the critical self-repairing size or due to a bone disease, such as degenerative scoliosis or osteoporosis. In these cases, it is necessary to use bone grafts to promote bone growth and recover from the damage [17].

Bone grafting is a procedure where new bone or a substitutional material is surgically introduced into a defect or around it to promote healing. Bone grafts are divided into four types according to the nature of the material. They can be autografts, that use the patient's own bone from another part of the body; allografts, if the graft comes from a donor; xenografts, if the bone comes from a different species; or synthetic bone grafts. Artificial bone grafts have been developed to make up for the disadvantages of using non-synthetic grafts, such as the risk of infection, immune incompatibility, pain, disease transmission or low supply. Furthermore, they serve for larger bone defects and there is always enough supply [17, 18].

Bone tissue engineering employs methods of manipulating several biomaterials to produce grafts that support cell growth. These grafts are scaffolds that gather physical, chemical and mechanical properties which are suitable for cell adhesion and new tissue formation [18].

The fundamental challenges in bone tissue engineering include selecting the most effective materials and level of porosity to use in scaffolds, achieving proper vascularization and host integration and evaluating the regenerated bone quality [19].

2.1. Scaffolds

A scaffold is a three-dimensional support used to provide a bioactive environment that mimics the extracellular matrix and therefore promotes cell adhesion and proliferation. Scaffolds can be used in the regeneration process of several tissues such as bone, nerve, muscle and others, and they must exhibit a level of porosity that allows the transfer of vital nutrients to cells, thus promoting blood vessel ingrowth [17, 18].

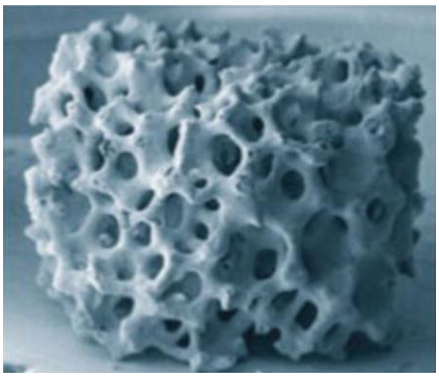

Figure 4: Calcium phosphate-based scaffold [20]

One of the challenges encountered by bone tissue engineering is building a satisfactory environment for tissue growth without rejection by the patient receiving the implant. Thus, the biomaterial can be implanted and afterwards it can be reabsorbed by the patient's body, being recognized by its immune system without triggering an exacerbated immune response. For this to occur, the materials used to produce scaffolds must be biocompatible so that they don't induce toxicity [21, 22].

The materials must be biodegradable, avoiding the necessity of surgical removal of the implant. They should degrade at a rate that is compatible to that of new tissue formation so that the scaffold can last until the renovation of the tissue takes place [23].

Other aspects that are important to evaluate in biomaterials are osteoinduction, osteoconduction and osteointegration. Osteoinduction stimulates osteogenesis, which is the process that recruits osteoblasts progenitors and induces them to proliferate and differentiate into osteoblasts. Osteoconduction is the formation of bone on the surface of the bone graft, which means it facilitates the migration and the adhesion of bone-forming cells across the scaffold leading to bone replacement. Osteointegration is the stable fixation of an implant directly onto bone [23-26].

Porosity is another crucial aspect to take into consideration when producing a scaffold, since it varies depending on where the scaffold will be placed (concerning the mechanical and cellular properties of the tissue) and on the patient's characteristics, such as their age, their disease or their nutrition. Porous and textured materials are widely used in medical device applications, since they allow interconnection between pores, thus supporting nutrient transfer and the migration and proliferation of cells. Porosity integrates the implant in a stable manner, reducing irritation in the surrounding tissue, which would otherwise be caused by micromotion. Additionally, textured materials improve tissue healing and increase vascularization of the host tissue. Pore size and pore conductivity influence the rate at which the forming bone grows. The

pore size of bones ranges from 10 to 100 μm. Previous studies in the area of bone tissue engineering have shown that, to achieve optimal cell attachment, pore size should be in the range of 100 μm to 150 μm. However, other studies have indicated that the required pore size for bone to successfully grow into the scaffold and to allow vascularization is between 150 μm and 900 μm. The level of porosity allowed in a scaffold is limited by the mechanical strength of the structure. The scaffold's level of porosity must be enough to allow cellular signalling and vascularization, but it shouldn't be too porous so as not to compromise the mechanical properties [27-29].

Scaffolds must exhibit mechanical properties that will allow them to withstand physical stresses in the patient's body, maintaining their structural integrity. At the same time, the material used in the scaffold must not exceed the strength of the surrounding bone, in the sense of avoiding stress shielding. So, it is desirable to match the mechanical properties of the scaffolds to those of bone. The tensile strength and Young's modulus of bone are demonstrated in table 1 [28, 29].

Table 1: Structural and mechanical properties of cortical and trabecular bone [30]

Bone Type	Porosity (%)	Young's Modulus (GPa)	Tensile Strength (MPa)	Compressive Strength (MPa)
Cortical bone	5 — 10	7 — 30	50 — 151	106 — 224
Trabecular bone	75 — 90	14 — 20	—	2 — 12

Grain size has been reported to have impact on cell adhesion. It refers to the size of each individual particle that composes the material of the scaffold. Studies show that cellular attachment and proliferation are favoured by smaller grain size, although the mechanism for why it is so is not yet clear. Nonetheless, due to studies demonstrating good cell behaviour in the presence of smaller grain sizes, scaffold fabrication processes are performed to form small particle structures [27].

The main aim of a 3D scaffold is to mimic the extracellular matrix, which is a composite tissue composed of macromolecules, such as collagens, proteoglycans, elastic fibres and cell-interactive glycoproteins. Collagens are responsible for the structural role of cells as they are the most abundant proteins in the human body. Proteoglycans are found within cells or at their surface, and their elastic properties allow cells to move and differentiate. Elastic fibres provide tissue flexibility. Lastly, cell-interactive glycoproteins are

specific amino acid sequences that are crucial for cell adhesion, since they work as adhesion recognition signals. Cells have surface receptors, as is the case of integrins, that are responsible for responding to the adhesion recognition signals and for linking extracellular proteins to the cytoskeleton within the cell. The proteins that are adsorbed to such receptors form a layer with which cells must interact with at the time of adhesion. The main role of extracellular matrix is to serve as a physiological substrate for cellular attachment, since most cells require a substrate where they can attach to in order to grow. In the same regard, in the presence of a scaffold, the protein layer works as a bridge between the cells and the artificial substrate [31].

The use of scaffolds for tissue engineering has a great advantage, that is the possibility to functionalize their surface for mechanical strength, degradation rate and cellular adhesion. Because most cell types exhibit increased activity and longevity when adhered to extracellular proteins, surface functionalization is an approach that can be used to immobilize bioactive molecules or biomimetic species on to substrate surfaces. This procedure can be performed, for example, on polymers that have reactive functional groups, which will allow the conjugation of peptides, growth factors or enzymes. However, studies have demonstrated that, in cases where the chemistry and topography of the surface of a material are optimal for primary cell adhesion, it does not mean that the conditions are also adequate for cell proliferation and differentiation. Therefore, the interaction between the substrate and cell is complex [27, 31].

Vascularization is a matter that calls for special attention in tissue engineering, since it is responsible for ensuring the transfer of nutrients for cell survival and differentiation. The process through which blood vessels grow is angiogenesis, and it is stimulated by angiogenic proteins such as growth factors. Growth factors can be incorporated in a scaffold to stimulate vascularization, as is the case of vascular endothelial growth factor (VEGF) and BMP-2. Wernike et al. [32] incorporated VEGF into calcium phosphate scaffolds for an *in vivo* test in mice and verified an increase in blood vessel density. However, they found that an excessive amount of VEGF leaded to malformed vasculature. BMP-2 stands for bone morphogenetic protein 2, being that BMPs are multi-functional growth factors that belong to the superfamily of the transforming growth factor beta (TGF-ß). In addition to heart, neural and cartilage development, BMPs also play an important role in bone formation. Indeed, BMP-2 finds application in therapeutic interventions related to bone defects, non-union fractures and osteoporosis, due to their ability to induce the differentiation of mesenchymal stem cells into osteoblasts [27, 33, 34].

Synthetic bone grafts are more effective and carry less infection risks than natural grafts, which encourages research on bone tissue engineering to focus on the most adequate properties for scaffolds, in order to mimic the extracellular matrix as similar as possible. The morphology, porosity and mechanical properties are important aspects to consider. In addition, the surface of scaffolds can be functionalized by adding bioactive molecules that improve cell adhesion or vascularization, for instance. Nevertheless, the choice of the biomaterial to be used is the most important factor for an efficient scaffold. It must be resorbable and biocompatible, and several ceramics and polymers have been studied for this purpose.

Chapter 3: Biomaterials

In tissue engineering, the challenge to find bone substitutes and implants that are long lasting stimulates the continuous search for materials that exhibit biocompatibility, biodegradability and good mechanical properties. Biomaterials may be natural, in the case of autografts, allografts and xenografts, or synthetic, which is the case of metals, polymers and ceramics [22, 35].

Based on their biocompatibility, materials can be classified as bioinert, bioactive and bioresorbable. Bioinert materials have minimum interaction with the surrounding tissues, which is the case of stainless steel, titanium, alumina and zirconia, for example. They are usually encapsulated by the body, through the formation of a fibrous capsule. Fibrous ingrowth is an inhibitor process of osteointegration. Once it takes place, bone formation will not occur due to the formation of the fibrous capsule that works as a physical barrier between the bone and the implant. For this reason, bone tissue engineering uses bioactive materials [17].

Bioactive materials interact with the surrounding tissues, where an ion-exchange reaction takes place between the implant and the surrounding environment and results in the formation of a biologically active carbonate apatite layer on the implant. This layer is chemically equivalent to the mineral portion of bone. Examples of bioactive materials are synthetic hydroxyapatite and bioglass [36].

Bioresorbable materials start to dissolve once they are inserted in the patient's body and are resorbed by specific cells. Common resorbable materials are tricalcium phosphate and hydroxyapatite. The difference between bioactive and bioresorbable materials is that the term "bioactive" is restricted to the surface of the material, whereas "bioresorbable" refers to their bulk [37].

3.1. Bioactive Ceramics

The main reason why bioactive ceramics are used in bone tissue engineering is their similarity to the natural bone matrix. They are hard, heat and corrosion-resistant, biocompatible and osteoconductive. However, there are some disadvantages associated with the use of ceramics in medicine, since they are brittle, so they can't bear mechanical efforts, and their porous structure increases the risk of fracture. Bioactive ceramics are widely applied in bone tissue engineering as they bond directly with bone without fibrous growth in-between. The main examples of ceramics used in this field are calcium phosphates and bioactive glasses [23, 25, 38].

3.1.1. Calcium Phosphates

Calcium phosphates are compounds containing calcium (Ca^{2+}), phosphorus (P^{5+}) and oxygen (O^{2-}) and are divided into different categories: orthophosphates (PO_4^{3-}), metaphosphates (PO_3^-), pyrophosphates ($(P_2O_7)^{4-}$), triphosphates ($(P_3O_{10})^{5-}$) and tetraphosphates (P_4O_{13}). The type of calcium phosphates that are applied in bone regenerating scaffolds are calcium orthophosphates, which exist in the inorganic part of bones and teeth. The efficacy of calcium phosphates in bone regeneration lays on the release of calcium and phosphate ions that increases ionic concentration and leads to more precipitation of more calcium phosphate. This facilitates protein adsorption and enhances bone formation [28, 38-40].

3.1.1.1. Hydroxyapatite (HA)

It is a hydrated calcium phosphate mineral that results from biomineralization in bone tissue formation and can also be synthesized for medicine. It belongs to the apatite family and its chemical formula is $Ca_{10}(PO_4)_6(OH)_2$. Synthetic hydroxyapatite has been widely used as a biomaterial due to its chemical formula and properties which are similar to those of the constituent of bone and since it enables bone cell proliferation. Its main application areas are dentistry and bone regeneration. It can be used either in bone grafts made entirely out of hydroxyapatite or as a coating [35, 41, 42].

The application of a hydroxyapatite-based implant carries several advantages, as it results in a good stabilization and fixation to the adjacent tissues, due to the formation of a biologically active carbonate apatite layer on the implant, typical of bioactive ceramics. Moreover, it is thermodynamically stable at pH 4,3 and above, which includes the physiological pH (7,4). It is osteoconductive but not osteoinducive. Despite these aspects, hydroxyapatite's low degradation rate comes as a disadvantage [24, 42].

3.1.1.2. β-Tricalcium Phosphate (β-TCP)

Tricalcium phosphates (of chemical formula $Ca_3(PO_4)_2$) are bioactive and bioresorbable materials. Compared to hydroxyapatite, they have greater solubility, which makes them less stable phases under physiological conditions and makes them have rapid resorption kinetics. This is an advantage considering that the long duration of hydroxyapatite can end up blocking the formation of new bone and result in stress concentration. Tricalcium phosphates lack osteoinductive properties, but exhibit good osteoconductivity [24, 43].

There are two polymorphs of tricalcium phosphates: α and ß. They have different crystal structures that depend on temperature. ß-TCP is stable below 1125°C, while α-TCP is stable above that temperature. α-TCP is more soluble than ß-TCP and forms calcium-deficient hydroxyapatite [38].

ß-TCP has been proven to support the attachment, differentiation and proliferation of important cells, like mesenchymal cells and osteoblasts, which makes it a good option for bone regeneration devices [44]. Its solubility stands in an almost balanced position between the rate of its absorption and the formation of new bone. Although some of its disadvantages lie on its poor mechanical properties, because it is brittle, ß-TCP is an excellent choice of material because of its unlimited availability and consistent quality [45].

3.1.2. Bioactive Glasses

Bioactive glasses typically consist of silicate, borate or phosphorus glasses with compositions based on SiO_2, B_2O_3 and P_2O_5, respectively, which are the three main glass-forming oxides. The most common bioactive glasses are silicate glasses. Bioglass 45S5 has been studied since 2006 and is considered the standard to which other bioactive glasses can be compared. They are biocompatible and have the ability to bond to bone and stimulate new bone growth. The reaction of the body upon implantation of Bioglass 45S5 is the formation of apatite and bonding to bone with release of calcium and silicon ions. The dissolution of SiO_2 forms silanol groups that gradually precipitate into a silica layer, which increases the migration of calcium and phosphate ions to form a layer of calcium phosphate. Bioactive glasses' properties such as osteoconduction and controlled resorbability make them very appealing for bone regenerating scaffolds. However, bioactive glass exhibits poor mechanical properties, which leads to its use in scaffolds within composite structures in order to take advantage of its ability to repair bone defects, or at the surface of an implant to facilitate the formation of apatite [39, 46].

Among the bioactive glasses with compositions based on the 45S5 composition, 13-93 and S53P4 designate the ones that have been most studied and applied. Other types of bioactive glass exist, and their structure differs according to their purpose. In silicate 45S5 or 13-94 glasses, the replacement of certain amounts of SiO_2 with B_2O_3 creates borate or borosilicate glasses with controllable degradation rate. The most studied borate glass has the designation of 13-93B3 and can be obtained this way. Phosphate glasses with CaO and Na_2O in their composition have also been subject of study. When compared to silicate glasses, they exhibit a faster degradation rate. Because their constituent ions calcium and phosphate are present in bone, phosphate glasses show great chemical affinity with bone tissue, which makes them

promising resorbable materials for bone tissue engineering [47]. Table 2 shows the nominal composition of the main bioactive glasses.

Table 2: Nominal composition (in wt%) of some bioactive glasses [46]

Oxide	Bioactive glass designation			
	45S5	**S53P4**	**13-93**	**13-93B3**
SiO_2	45,0	53,0	53,0	0
Na_2O	24,5	23,0	6,0	5,5
CaO	24,5	20,0	20,0	18,5
P_2O_5	6,0	4,0	4,0	3,7
K_2O	0	0	12,0	11,1
MgO	0	0	5,0	4,6
B_2O_3	0	0	0	56,6

Studies performed by Hoppe et al. [48], Hebibovic and Barralet [49] and Lakhkar et al. [50] have shown that both borate glass 13-93B3 and silicate glass 45S5 doped with metal ions such as Cu, Zn and Sr stimulate angiogenesis or osteogenesis, which is of interest for bone tissue engineering. The release of the ions happens as the glass converts to hydroxyapatite [46].

When choosing the adequate bioactive glass to be used in a porous scaffold for bone regeneration, one important aspect to bear is the degradation rate and bioactive potential of the glass (conversion of the glass to hydroxyapatite). The degradation rate of the glass depends on its composition. For example, a 13-93 glass has a higher SiO_2 content than a 45S5 glass, therefore its degradation and conversion to hydroxyapatite is slower. In fact, studies performed *in vitro* [47] and *in vivo* [51] have shown that borate 13-93B3 glass degrades up to ten times faster than silicate 13-93 glass. The glass's composition also influences its biocompatibility, because the ions that are released during its degradation affect the environment where the glass is placed by changing the ionic concentration and pH. Moreover, the glass's mechanical properties much match the ones of bone as much as possible. Silicate glasses such as 13-93 typically exhibit higher strength and elastic modulus than borate glasses, as is the case of 13-93B3. Because of its slower degradation rate, the 13-93 silicate glass maintains its strength in an aqueous phosphate solution more than the 13-93B3 borate glass [46].

In terms of processing the bioactive glass into a three-dimensional porous scaffold, it is fundamental to consider the existing risk of crystallization. Silicate 45S5 glass undergoes crystallization during sintering and this will limit the sintering of the glass's particles into a dense phase, which results in poor compressive strength of the scaffold and therefore makes it suitable for low-load defect places only. Silicate 13-93 glasses, however, exhibit a larger interval between their glass transition temperature and their onset crystallization temperature, which makes it easier to sinter them into scaffolds with a dense phase and therefore higher strength, as shown in a study performed by Xiao et al. [52] in 2016 [46].

3.1.3. Bioactive Ceramic Composites

The solubility of calcium phosphates in aqueous solution is an essential property to take in consideration and is dependent on the calcium/phosphorus ratio. In general, the higher the Ca/P ratio, the lower the solubility. As it is demonstrated in table 1, ß-TCP is much more soluble than hydroxyapatite, possibly leading to loss of scaffold integrity earlier than desirable. In the same extent, hydroxyapatite's slow degradation rate can interfere with new bone formation. Thus, one way to achieve the desired solubility in the final product is to combine both materials, obtaining biphasic calcium phosphates (BCP). The higher the proportion of ß-TCP, the higher the solubility of the combination. Moreover, the lack of mechanical resistance exhibited by ß-TCP can be overcome with the presence of hydroxyapatite [53, 54].

Table 3: Properties of some calcium phosphates [54]

Compound	Formula	Ca/P ratio	Solubility at 37 ºC (mg/L)
HA	$Ca_{10}(PO_4)_6(OH)_2$	1,67	$9,6 \times 10^{-5}$
ß-TCP	ß-$Ca_3(PO_4)_2$	1,5	0,15
α-TCP	α-$Ca_3(PO_4)_2$	1,5	0,24

Houmand and his team [55] studied the *in vitro* behaviour of scaffolds with different proportions of HA/ß-TCP (100:0, 60:40, 20:80) in both water and simulated body fluid and verified that the scaffold with the greatest amount of ß-TCP showed the fastest dissolution rate. Jensen et al. [56] performed a similar study on the *in vivo* behaviour of scaffolds in the mandibles of minipigs, using HA/ß-TCP ratios of 20/80, 60/40 and 80/20, and concluded that the amount of bone formation and degradation of scaffold material was higher with

the 20 HA/80 ß-TCP ratio. Additionally, Sulaiman at al. [57] tested scaffolds composed of only hydroxyapatite and mixing hydroxyapatite with ß-TCP and reported that both compositions resulted in cell proliferation and bone formation. However, gene expression of type I collagen was more remarkable with the HA/ß-TCP construct, leading to the conclusion that the mixture composition has better osteogenic potential than solely hydroxyapatite. Because ß-TCP is a greater source of calcium, it favours bone mineral deposition in a more successful way than hydroxyapatite [58].

Bioglass 45S5 can also be combined with other materials in order to overcome its main limitations. The challenge of using it in porous scaffolds is connected to it undergoing crystallization when sintered, in addition to the fact that, similarly to hydroxyapatite, its slow degradation rate may complicate resorption and formation of new bone [39]. The teams of Ruiz-Aguillar [59] and Badr-Mohammadi [60] produced scaffolds composed of ß-TCP and a phosphate-based bioglass, and scaffolds composed of a biphasic calcium phosphate (HA and ß-TCP) with a bioglass based on $64SiO_2–31CaO–5P_2O_5$, respectively. Both studies obtained scaffolds with good mechanical properties and the performed *in vitro* tests exhibited good cell adhesion and proliferation.

3.2. Polymers

Another great choice of biomaterials to be used in scaffolds are polymers, mainly due to the versatility of their chemistry and their mechanical properties. In fact, it is the type of material that is most intensively investigated for biomedical uses. Polymers divide into natural and synthetic polymers. The latter can be produced under controlled conditions so that their properties, such as tensile strength, porosity and degradation rate can be predefined for specific applications. This control also allows this type of material to be produced in large quantities with uniform properties and to have a long shelf life. Natural polymers were amid the first materials studied for clinical use due to their biological properties. They have better interactions with different cell types with a lower risk of immune responses. However, their availability is much more limited, as well as the control over their properties. Even though synthetic polymers are associated with the risk of toxicity, they are cheaper and have better functionality [9, 61].

The efficacy of the scaffold in respect to new tissue formation is directly related with the characteristics of the substrate, since it affects cell behaviour and function once cells adhere to it. This is the reason why controlling synthetic polymers' properties is so important and why they are mostly used. This type of materials' capability for chemical modifications makes it possible to alter their properties by changing their architecture using a linear, branched or

comb-shaped polymer or varying the functional group. The modification of the polymer combination through the preparation of a physical mixed polymer or a chemically bonded copolymer is also feasible. Because of this, it is possible to define and monitor the behaviour of the scaffold in terms of its degradation rate and cell-material interaction once it is implanted. Critical characteristics of the surface of the material include wettability, charge, roughness and rigidity. Because cell adhesion depends on the presence of serum, it is necessary to have a degree of wettability that allows the adsorption of proteins such as fibronectin to the material. In this regard, an intermediate contact angle has been proven to be optimal for proteins to adsorb to the surface. In addition, positively charged surfaces increase cell adhesion in the absence of a serum. Roughness is another significant factor, as cells find the peaks of a rough surface as anchorage points to which they can attach [31].

The biodegradability of a polymer must ensure that the scaffold is completely degraded and eliminated as the need for its support diminishes, so that potential reactions that may arise from its long-term presence can be avoided. The degradation of the scaffold occurs through hydrolysis and/or enzymatic processes, which result in tissue-compatible metabolites that can be used in the carbohydrate or protein metabolism. Water and carbon dioxide are examples of resulting breakdown products that are eventually excreted in urine or faeces [31].

Once a polymeric scaffold is implanted, cellular mechanisms are activated to begin inflammation and healing mechanisms. The extent of these mechanisms is related to the implant's size, shape and physical properties, and it can usually be divided in three phases. Phase I consists of the initiation, resolution and organization of an acute and inflammatory response. During this phase, inflammatory cells denominated monocytes prevail in the implantation site, where they differentiate into macrophages. In phase II, the material begins to undergo macrophage phagocytosis until its complete degradation. As the polymer degrades, there is a divergence from what would be the optimal wound-healing condition, originated by the release of oligomers and monomers that result in the formation of particulates. The degradation rate of the polymer is what defines this phase's duration and the polymer's biocompatibility. As the immune response progresses, macrophages coalesce and form fibrous capsules. Phase III is characterized by the acceleration of the degradation process by the macrophages and the fibrous capsule enhancement. The migration of more cells ensues, with the void generated by the loss of the implant being occupied by them and neovascularization proceeds [31].

3.2.1. Synthetic Polymers

Currently, the most common synthetic bulk resorbable polymers used in scaffolds are poly(L-lactic acid) (PLLA), polyglycolic acid (PGA), polycaprolactone (PCL) and poly(lactic-co-glycolic acid) (PLGA). These synthetic polymers are often combined with natural ones, that improve hydrophilicity, cell attachment and biodegradability [27].

Poly(lactic-co-glycolic) acid (PLGA) is the copolymer of PLA and PGA, as its repeat unit consists of lactic acid (LA) and glycolic acid (GA) monomers. It undergoes hydrolytic degradation when in contact with body fluids, which makes it biodegradable. Its hydrophilicity/hydrophobicity and its crystallinity can be tailored by changing the LA/GA ratio. The existence of an additional methyl group in lactic acid makes PLA more hydrophobic than PGA, and consequently more slowly degradable. Despite PGA's fast rate of degradation, it lacks strength. Although the byproducts of PLGA's degradation (lactic acid and glycolic acid) are nontoxic, their highly acidic nature in large quantities can be problematic for the body to metabolize rapidly. This can be prevented by changing the PGA:PLA ratio to a greater quantity of PGA, leading to a slower degradation rate and therefore a slower release of acidic byproducts at once. In this regard, it is necessary to find an equilibrium so that the degradation rate is slow enough to limit the released byproducts but fast enough that the implant won't last too long in the individual's body [27].

Poly(L-lactic acid) (PLLA) is one of the enantiomeric forms of PLA, being the other one poly(D-lactic) acid (PDLA). It is a biodegradable polyester that results from the polymerization of L-lactide. Although its degradation rate is faster than polycaprolactone's, it is still considered slow in comparison with other polymers used in tissue engineering. The byproducts of PLLA degradation are mostly non-toxic. However, its degradable fragments are highly crystalline, which can lead to inflammation in the body. For this reason, it its often blended with other polymers like L-lactic acid or D, L-lactic acid, because not only are they less inflammable due to their lower crystallinity, but they also have a rapider degradation rate [27, 62].

Poly-D,L-lactic acid (PDLLA) is the result of adding D-isomers into an L-isomer based polymerization system of PLA. PDLLA's biomechanical, thermal, rheological and biological properties can be tailored through different proportions of the two isomers. D-isomer is characterized by a rapid resorption and low crystallinity, whereas L-isomer is the opposite. The material's rheology is also dependent on the amount of L-isomer, since its high crystallinity causes a high shear viscosity. The glass transition temperature also increases with increasing content of L-isomer [63].

Polycaprolactone (PCL) is a polyester that consists of nonpolar methylene groups and one semi-polar ester group. It can be processed into porous structures and exhibits great biocompatibility. Although it degrades through a hydrolytic reaction under physiological conditions, its high crystallinity and hydrophobicity cause its slow degradation. A study carried out by Sun et al. [64] testing the long-term *in vivo* degradability of this material in rats showed that PCL capsules are able to remain mechanically intact for two years. For this reason, this material is more promising for long-term implants and drug delivery systems than for scaffolds. Its poor mechanical strength and low bioactivity are also limiting factors for its use in bone regenerating scaffolds. However, there are several approaches that aim to improve its bioactivity, which include copolymerization and surface functionalization. Moreover, PCL can be combined with other polymers or bioactive ceramics, overcoming the lack of suitable properties of PCL alone [27]. Table 4 presents some properties of the aforementioned polymers.

Table 4: Physical properties of synthetic polymers [65, 66]

Polymer	Melting Point (°C)	Glass Transition Point (°C)	Biodegradation Time (months)	Tensile Strength (MPa)	Young's Modulus (GPa)
PLGA	Amorphous	45 — 55	Adjustable: 1—12	41,4 — 55,2	1,4 — 2,8
PGA	220 — 233	35 — 45	6 — 12	60,0 — 99,7	6,0 — 7,0
PLA	150 — 162	45 — 60	36 — 60	21,0 — 60,0	0,3 — 3,5
PLLA	170 — 200	55 — 65	> 24	15,5 — 150,0	2,7 — 4,2
PDLLA	Amorphous	50 — 60	12 — 16	27,6 — 50,0	1,0 — 3,5
PCL	58 — 65	(-72) — (-60)	> 24	20,7 — 42,0	0,2 — 0,5

3.2.2. Natural Polymers

Collagen and hyaluronic acid are two polymers that naturally occur abundantly in the human body, which ensures their biocompatibility. Type I collagen is the fibrous protein that makes up 90% of the organic portion of bone. Among the various types of collagen that exist, types I, II, III, V and XI have been tested for both soft and hard tissue engineering applications. Out of these, type I is considered the "gold standard" due to the minor immune response risk it carries. It is naturally part of the extracellular matrix, which makes it inherently biocompatible, biodegradable and it stimulates cell proliferation and differentiation. The surface of collagen scaffolds usually exhibits a rough morphology that contributes to the structure's fibrous nature and porosity.

Nonetheless, there is a difficulty concerning their fabrication, that is associated with significant alterations to the integrity of the structure. In addition, the choice of a technique to produce collagen scaffolds is limited, because although it is possible to synthesize collagen scaffolds via several techniques, many of them alter the material's surface, and cellular attachment on collagen is very much dependent on the surface morphology [27, 67].

Hyaluronic acid is a glycosaminoglycan that takes part in mediation of cellular signalling and regulation of cell adhesion and proliferation. It is found mostly in connective, epithelial and neural tissue. Hyaluronic acid has a slow degradation rate and can be used for both soft and hard tissues regeneration. Its popularity is partly due to the number of fabrication techniques through which it can be processed, enabling its use for nearly any tissue regeneration application [27].

Another example of a natural polymer that can be applied in scaffolds is chitosan. It is a biodegradable polysaccharide that derivates from chitin. Usually obtained from shrimp shell, it has been reported to be biocompatible because it can be degraded by lysozyme, which depolymerizes the polysaccharide. It has great bioactivity, as it is able to promote cellular adhesion without any need for functionalization. As a biomaterial, chitosan has limited solubility at physiological pH, which gives it a slow degradation rate [27].

Silk is a natural polymer that can be extracted from worms and insects, and its contribution in the tissue engineering field is due to its great cellular adhesion properties. Before being used, silk must be cleansed in order to remove a toxic component named sericin and obtain the remaining component fibroin. Fibroin displays a relatively high tensile strength and good biocompatibility, making it promising for the regeneration of various tissues, such as bone, cartilage, nerve and tendon [27].

3.2.3. Polymer Composites

Like ceramics, different polymers can also be combined with the aim of gathering different properties. Such combinations are most interesting when it comes to mixing natural and synthetic polymers, since synthetic polymers can provide mechanical strength and other specific characteristics that can be defined in their processing, and natural polymers contribute by granting an adequate environment for cell activity [27].

Sheik et al. [68] produced PLGA and PLGA/silk scaffolds that combined the degradation rate of PLGA with the hydrophilicity of silk. The scaffolds were produced using a freeze-drying method and implanted in rat calvariae over a period of four weeks and concluded that the incorporation of silk resulted in

improved hydrophilicity, mechanical properties and viability of osteoblasts. Bhattacharjee's team [69] produced electrospun PCL/silk scaffolds in different ratios and found that the specimens with a greater amount of silk showed increased cell attachment and superior extracellular matrix formation, in addition to better cytocompatibility.

Another polymer/polymer combination was produced by Mano et al. [70], consisting of PLLA/chitosan scaffolds with 0%, 0,5% and 1% of chitosan in their composition. The *in vitro* study demonstrated that the structures with a higher chitosan content resulted in an increased apatite deposition. Antunes et al. [71] mixed hyaluronic acid with PLLA in their *in vitro* study. They verified that the surface roughness of the scaffolds was increased by the presence of hyaluronic acid and that their mechanical properties were acceptable for bone tissue engineering. However, they concluded hyaluronic acid concentrations higher than 0,1% can inhibit cells viability.

3.3. Polymer/Bioceramic Composites

Although different bioactive ceramics and polymers have been studied for bone tissue regeneration, there is not a material that meets all the requirements to fabricate an ideal scaffold. For instance, despite the similar composition of calcium phosphates and bone, ceramics are not very mechanically resistant. In the same extent, polymers confer structural resistance to scaffolds, but their surface properties may be inadequate for cell adhesion and require surface treatments, which can be expensive and time-consuming. A step towards the production of more successful scaffolds is the use of different materials combined, in order to blend the advantages of each of them. Thereby, the composite materials that result from these combinations consist of a polymeric phase that confers toughness and compressive strength and a ceramic phase with bioactivity that will improve the degradation rate. Furthermore, considering the natural structure of bone tissues as multiphase nanocomposites, it becomes relevant to use composite materials that mimic tissues more satisfyingly. Nanocomposites are of great interest due to their similarity to bone tissue [9, 72].

Concerning the fabrication of composites, it is necessary to ensure that the components are mutually chemically stable, and that the final combination is sufficiently strong for the desired application. Another important aspect is the thermal expansion coefficient of the components, that should match. Most studies on composite materials for scaffolds focus on ceramic-polymer composites, being usually the ceramic the dispersed phase in a polymer matrix [9].

Numerous combinations have been studied. Cao et al. [73] produced composite scaffolds of PGA/β-TCP (with a weight ratio of PGA:β-TCP equivalent to 1:3) using the solvent casting and particulate leaching method. They prepared a solution of PGA in highly fluorinated organic solvents, in which PGA is soluble due to its high crystallinity. β-TCP powder was mixed in the solution along with NaCl crystals as a porogen. The scaffolds were tested in femoral defects in rats over 90 days after surgery and exhibited a strong ability for mineralization, osteogenesis and biodegradation. Konopnicki and her team [74] also used β-TCP in their study. However, they mixed it with PCL instead and 3D-printed scaffolds that were tested *in vivo* in pig mandibular bone, over a period of eight weeks, resulting in bone formation and good bone penetration into the scaffolds.

The teams of Park [75] and Mohseni [76] produced composite scaffolds of β-TCP with natural polymers, both using the freeze drying technique. The first study consisted of silk fibroin/β-TCP scaffold tested in rats with a calvarian defect. The second study was on collagen/β-TCP scaffolds implanted in femur bone defects in rabbits. Both demonstrated good results in terms of biocompatibility, osteoconductivity and mechanical properties.

Bioglass can also be integrated in composite scaffolds, which is the case of the study performed by Ródenas-Rochina et al. [77], who compared PCL/HA and PCL/bioglass scaffolds. Both composites were studied *in vitro* during 28 days, demonstrating acceptable mechanical properties, high interconnected porosity and cell proliferation. Other examples of experimented combinations include collagen/HA [78, 79], collagen/bioactive glass [80], chitosan/bioactive glass/PLGA [81], HA/PLGA/bioactive glass [82], PLLA/bioactive glass [83], silk/HA [84, 85], hyaluronic acid/HA [86] and PLGA/HA [87].

For the purpose of bone regeneration grafts, it is important that the materials that compose scaffolds are bioresorbable, so that the structure biodegrades at a rhythm that is compatible to it not being necessary anymore. They should also be bioactive in order to react with body fluids and produce an apatite layer as they bond directly to bone and enhance bone tissue formation. Ceramics and polymers that meet these requirements are several; however, the rate at which a material degrades, its mechanical strength or its surface properties may be not be sufficient for an efficient behaviour in a scaffold, leading to research on composite materials, either ceramic/ceramic, polymer/polymer or ceramic/polymer. Along with the different materials that have been studied throughout the years, so have various techniques that can process them and lead to the construction of scaffolds.

Chapter 4: Scaffold Production Techniques

There are numerous methodologies to produce ceramic and polymeric scaffolds. However, there are some factors to consider when choosing a technique with which to proceed. It must not alter the chemical properties of the materials in any way that can prejudice their clinical use, as well as their biocompatibility. Some techniques are only appropriate for a group of materials. The techniques should lead to interconnected pores with regular size, distribution and morphology, and should have good reproducibility [88].

Scaffolds production techniques are divided into conventional and advanced. Conventional methods result in random architectures within the scaffold. Advanced methods allow the control of the structure shape, including pore size and geometry, and they result in improved mechanical properties that can vary in order to become adequate for either soft or hard tissues. However, they require more expensive equipment than conventional methods [20, 89].

4.1. Conventional Methods

4.1.1. Replica Technique

It consists of impregnating a template with a ceramic suspension, being calcium phosphates the often-used materials for this method. The template can be natural, like wood or coral, or synthetic, like a polymer, as is the case of polyurethane. Once the suspension dries up, the template is removed, creating a replica of the original template structure. Via the replica technique, it is possible to obtain total open porosity levels between 40% and 95% in a structure with highly interconnected pores with sizes between 200 μm and 3 mm [20, 90].

4.1.2. Sacrificial Template

This process involves the preparation of a biphasic composite with ceramic particles that constitute the matrix and a dispersed phase that is homogeneously distributed in the matrix serving as a template that will afterwards be extracted and generate pores. This substance can be for example, polyvinyl alcohol. The sacrificial template method is opposite of the replica method, as it creates a negative version of the original template [20, 89].

4.1.3. Direct Foaming

A foam is formed through the inclusion of gas bubbles in a ceramic suspension. After the slurry dries up, the bubbles result in spherical pores, and the material can then be sintered to result in a high-strength structure. The final porosity depends on the amount of gas that is incorporated into the slurry. A foaming agent can be used to form the bubbles in the suspension, and it is posteriorly removed by the sintering process [20].

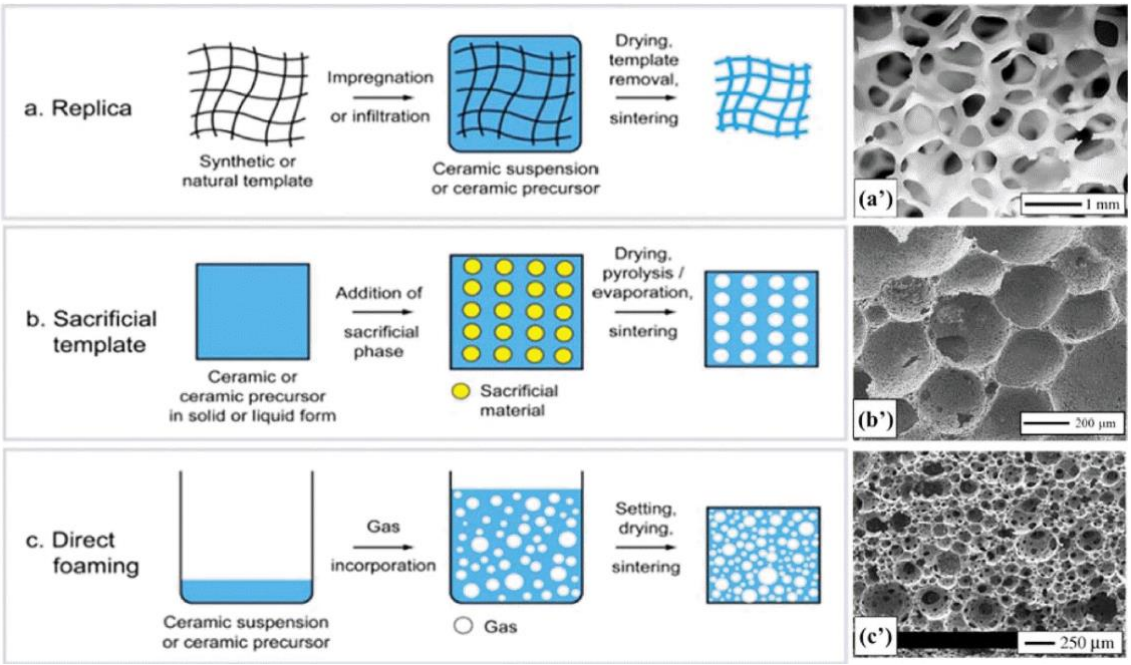

Figure 5: Scheme of the procedures and pore sizes obtained with the a) replica technique, b) sacrificial template and c) direct foaming [91]

4.1.4. Freeze Drying

Freeze drying can be applied to both ceramics and polymers, including hydroxyapatite, ß-TCP, PLGA, PLLA, PGA, collagen and chitosan. It consists of producing a ceramic or polymeric slurry, pouring it into a mold and freezing it. The growing ice crystals of the solvent will form the pores, and once the slurry is solidified, it will undergo lyophilization. The sublimation of the frozen solvent results in a highly porous scaffold with low stiffness. It is important to avoid a quench freezing process that will not give enough time for ice crystals to grow. Nevertheless, the resulting pores are small, which is a disadvantage, along with a low pore size distribution and a long processing time. The fact that high temperature or a leaching step are not required comes as an advantage [20, 89].

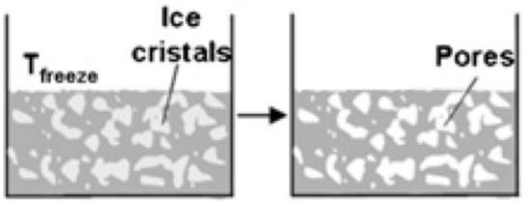

Figure 6: Scheme of the freeze-drying technique [88]

4.1.5. Solvent Casting and Particulate Leaching

This method is meant to be performed on polymers, such as PLLA, PLGA and collagen, or polymers combined with ceramics. It is a simple and inexpensive method where a polymer is dissolved in an organic solvent and the solution is mixed with water-soluble particles that will act as a porogen (NaCl salt, for example). The solvent is evaporated, leaving behind a polymer matrix with salt particles. Then, the salt is leached out with water or another solvent that leaches out only the porogen, forming a porous scaffold. The pore size of the final structure corresponds to the size of the porogen particles and can, therefore, be defined. It is possible to obtain a porosity of 93% with an average pore size of 500 μm. However, the interconnectivity between pores is low and hard to control, as well as the morphology of pore walls. Although this is a very easy method to proceed with, the use of organic solvents is a major drawback, since they may leave residues that are toxic [31, 88].

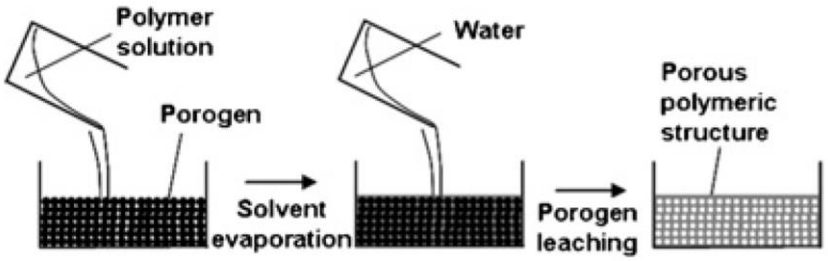

Figure 7: Scheme of the solvent casting and particulate leaching technique [88]

4.1.6. Gas Foaming

This is a technique meant to be applied with polymers or polymer/ceramic composites. It consists of exposing polymer disks or pellets (for example, PLLA, PLGA, collagen) to high pressure gas, in order to saturate the polymer. Afterwards, the gas pressure is reduced, leading to nucleation of gas bubbles in the polymer matrix. The porogen that is often applied in this procedure is CO_2, due its non-toxicity and non-inflammability. The big advantage of gas foaming

is that it does not require using organic solvents nor high temperatures, thus avoiding toxic residues and damage of the materials [88, 92].

4.1.7. Phase Separation

The concept of this method is to have a homogeneous multicomponent polymer solution and then create a thermodynamically instability that will provoke the separation of the different phases, as a consequence of the solution lowering its total free energy. The most common procedure is to thermally induce the phase separation. It begins with the dissolution of a polymer in a solvent at an elevated temperature, close to the melting point of the polymer, in order to form a homogeneous melt-blend. Subsequently, the solution is cooled in a controlled manner to separate the solution into a polymer-rich phase and a solvent-rich phase. The latter is then removed through evaporation or sublimation, leaving the polymeric matrix to dry and create a porous structure. The advantage of such technique is the elimination of a leaching step to remove a porogen. However, it is not possible to precisely control the morphology of the scaffold [67, 88, 92]. This technique allows the preparation of polymer and polymer/ceramic scaffolds like PLGA/hydroxyapatite [93].

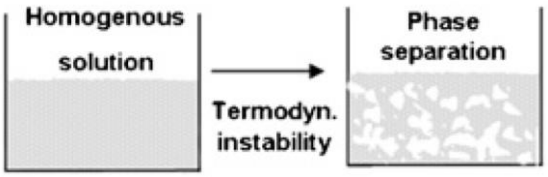

Figure 8: Scheme of the phase separation technique [88]

4.1.8. Melt-Quenching

Melt-quenching is the main technique used to process bioactive glass. It consists of melting the chosen oxides that will compose the bioactive glass (such as oxides of silica, calcium, phosphate or sodium). The melted mixture is quenched in a graphite mould or in water, forming a frit that is subsequently grinded to powder. In order to produce bioactive glass scaffolds, this technique can be followed by, for example, the replica technique, by mixing the glass powders with a binder (polyvinyl alcohol, for instance) and impregnating a polymeric template, which is lastly removed via a thermal treatment [94, 95]. An alternative was presented by Brovarone et al. [96], who mixed the glass powders with polyethylene particles and uniaxially pressed the mixture to obtain a green compact. The compact was sintered in order to remove the polymer particles and obtain a porous structure.

4.2. Advanced Methods

Rapid prototyping is a set of techniques that can quickly produce a scale model of a part or an assembly using CAD (Computer Aided Design) technologies. The production of the parts is done via additive manufacturing, which means that the material is deposited layer by layer into the desired architecture. It is possible to adapt the shape and size of the scaffold to match the patient's bone concerning the condition and location of the surrounding bone. Rapid prototyping techniques allow the production of highly complex and reproducible structures [20].

4.2.1. 3D Printing

3D printing enables the fabrication of both polymeric and ceramic scaffolds, like PCL [97] and tricalcium phosphate [98]. The material to be deposited is a binder solution, and the inkjet head of the device prints drops of it onto a powder bed. The layers are deposited on top of each other and merge at the same time of the material deposition. This powder bed serves as a support for each layer and is removed once the printing is finished. [20, 89].

4.2.2. Stereolithography

This process uses a vat of curable liquid photopolymer resin and an ultraviolet laser beam. The laser beam solidifies the resin through a movement that is defined by a pre-programmed design, and when a layer is complete, the lifting platform where the vat is inserted lowers one level so that a new layer can be solidified. Once the final product is fully printed, it is washed with a solvent to remove wet resin off its surface [20]. Using stereolithography, it is possible to create not only polymeric and ceramic scaffolds (for example, hydroxyapatite [99]), but also polymer/ceramic composites, such as ß-TCP/collagen [100].

4.2.3. Electrospinning

A solution of polymeric materials or ceramic/polymer composites is subjected to an electric current that creates electrostatic forces greater than the surface tensions of the solution. This results in the formation of a solid fibre that is continuously elongated due to the electrostatic repulsion between the surface charges and the solvent evaporation. The process is controlled by two electrodes with opposite polarity electric charges. One of them is placed in the polymer solution and the other in the fibre collector. As the polymer solution is ejected and creates a fibre that is deposited on the collector, the solvent

evaporates. The characteristics of the fibre depend on the viscosity and molecular weight of the polymer used. Numerous polymers can be used in this technique, such as silk fibroin, collagen and chitosan. It is possible to obtain high surface-to-volume ratios, good mechanical properties and high porosity through electrospinning [20, 101, 102].

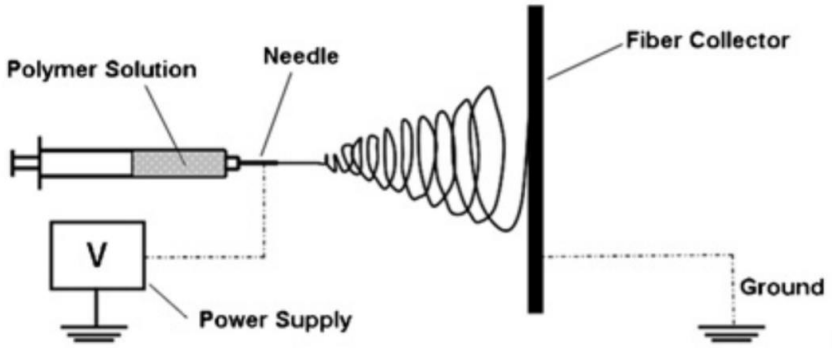

Figure 9: Scheme of the electrospinning technique [88]

The aforementioned techniques have varied precisions, that result in scaffolds with very different morphologies. Using conventional methods, it is complicated to control the porosity of scaffolds; however, it is possible to do so within a small range of pore size, as is the case of direct foaming and freeze-drying techniques. Replica technique, sacrificial template and direct foaming are mostly appropriate to produce ceramic scaffolds, whilst other techniques allow the creation of both polymeric and ceramic scaffolds, and composite structures. Although some techniques are restricted to the materials that can be processed using them, they may be simpler to perform and cheaper. Nonetheless, advanced techniques enable the fabrication of controlled-shape structures using different types of materials, making it possible to choose pore size, pore geometry, spatial distribution of pores and interconnectivity.

Chapter 5 - Magnetic Biomaterials

Biomaterials can be stimuli-responsive, meaning that they are able to react to external signals. In this regard, magneto-responsive materials can respond to external magnetic fields and this fact makes them promising materials for both diagnosis and therapeutic applications, such as magnetic resonance imaging, drug delivery systems, tissue regeneration and others [103].

In fact, magnetism has already been integrated into several areas of medicine with FDA approval [104]. For example, therapeutic hyperthermia is a cancer treatment that consists in locally increasing the body temperature, based on malignant cells and their specific vasculature reduced heat tolerance. Magnetic hyperthermia is already being applied as it enables a localized heating of tissues. It is a concept that consists of injecting magnetic nanoparticles directly into tumours and then applying an external oscillating magnetic field that induces oscillations of the nanoparticles' magnetic moments, generating heat [105]. The NanoTherm® therapy is based on this concept, for the treatment of brain tumours. It uses a liquid that contains iron oxide particles with a diameter of 15 nm that is injected directly into the tumour. Afterwards, the patient is subjected to an alternating magnetic field that changes its polarity up to 100 000 times per second, causing the nanoparticles to generate heat and provoking the death of the cancer cells [106].

Drug delivery systems can likewise take advantage of magnetism in order to face its two main challenges: targeting the drug to a specific site and having the ability to control drug release over time. Through the use of magnetism, the amount of drug that is required to achieve a certain concentration in the desired site is reduced and possible side effects are minimized due to less non-target sites being in contact with the drug. In this regard, the method of magnetically guided drug targeting (MGDT) involves conjugating the drug with magnetic nanoparticles (MNPs) and administering this complex via intravenous or intra-arterial injection. Then, external magnetic fields are generated using permanent magnets to target the complex to the desired site and concentrate it there. Chemotherapeutic treatments for cancer have the great disadvantage of being non-specific, leading to a general systemic distribution and consequently affecting healthy cells, which is why MGDT is applied in this field. However, an external magnetic field guides the particles to where the field is maximum, that is at the body surface, which complicates the guidance of the drug to deep tissue. Thus, another possible approach is to implant a magnetic scaffold in the site where the drug must be delivered that will work as a target to attract the magnetic nanoparticles conjugated with the drug and allows the control of their space distribution [103, 107, 108].

Magnetic nanoparticles also take part in *in vivo* diagnostic imaging, helping diagnose diseases at their earliest stages or even prior to their manifestation. Magnetic resonance imaging (MRI) is a field where this concept is being developed. Although MRI is a diagnosis technique that provides detailed images of organs and tissues, the sensitivity of its probes is limited, and can be enhanced by contrast agents. Gastromark™ is a product that puts this concept into practice in bowel MR imaging. It consists of siloxane-coated superparamagnetic iron oxide particles, a complex denominated Ferumoxsil, intended for oral administration. Once ingested, Gastromark™ takes approximately 45 minutes to fill the stomach and small intestine, after which the MRI procedure can be initiated. MRI uses the contrast of protons (hydrogen nuclei) to create images of tissues. However, there are situations where the contrast between healthy and diseased tissues is not enough and therefore require such contrast agents [109, 110].

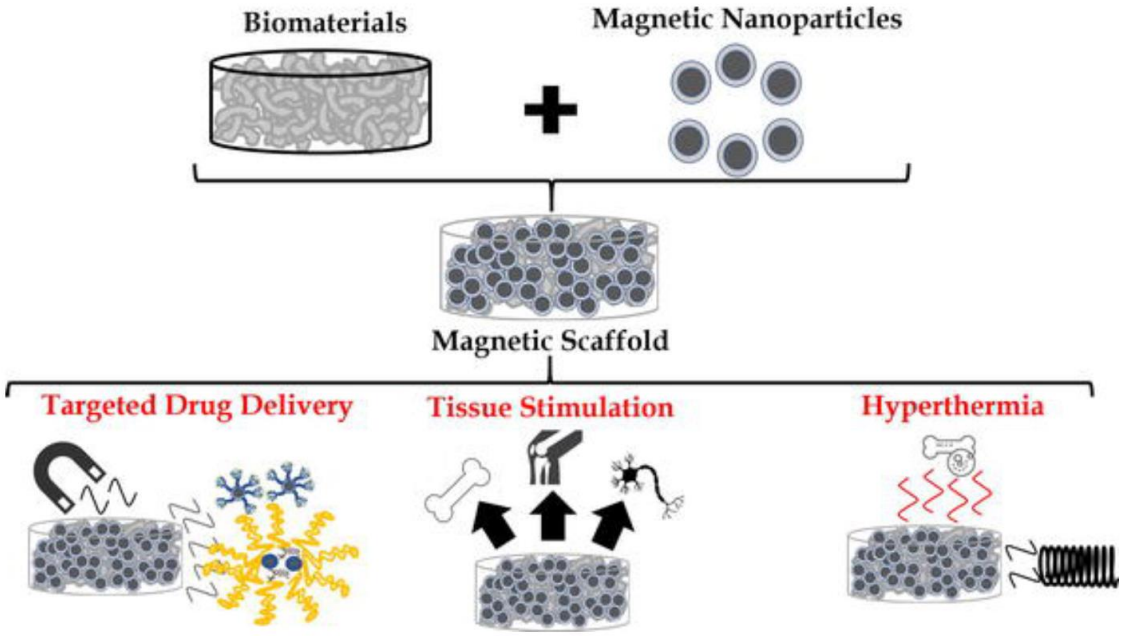

Figure 10: Examples of potential biomedical applications of magnetic biomaterials [103]

In the same regard, the tissue engineering field has been synthesizing and characterizing magnetic scaffolds to analyse their influence in tissue regeneration. Magnetic scaffolds have been investigated for the repair and regeneration of cardiovascular tissue, neuronal tissue, bone and cartilage. Bone is the most studied tissue because it naturally requires continuous mechanical stimulation, which can be delivered by magneto-responsive biomaterials [103].

5.1. Magnetic Nanoparticles

Nanotechnology is an area that enables the manipulation of materials at cellular and molecular levels, thus leading to great advances in several areas of science and technology including healthcare, electronics, information technology and others. A material is considered nanometric when its structural features have dimensions between 1 and 100 nm in at least one direction. In biomedicine, it is important to match the size of biomaterials with the size of cells (10-100 nm), proteins (3-50 nm), genes (10-100 nm), etc, so that those materials are well integrated and normal functions of the body are not disrupted. Furthermore, nanoscale materials can reach places that are inaccessible to greater scale materials [107, 111].

Among the different metals that can constitute magnetic nanoparticles, iron oxide nanoparticles (IONPs) are the only appropriate for clinical use, according to US Food and Drug Administration. This is due to iron's natural existence in the human body, as is the case of ferritin in myoglobin and hemoglobin, which ensures that pre-existing metabolic pathways are efficient to process the iron oxide nanoparticles. IONPs' advantages lay on their high magnetic susceptibility, very low toxicity, chemical stability, biodegradability, ease of synthesis and less sensitivity to oxidation [112, 113].

5.1.1. Iron Oxide Nanoparticles

The most common forms of iron oxides existing in nature are magnetite (Fe_3O_4), hematite (α-Fe_2O_3) and maghemite (γ-Fe_2O_3). Hematite is a semiconductor anti-ferromagnetic iron oxide. Its high resistance to corrosion and low cost justify its wide application in catalysts, pigments and gas sensors [114]. On the contrary, magnetite and maghemite are suitable for biomedical use. Maghemite is a less thermodynamically stable polymorph of hematite and naturally occurs by topotactic oxidation of magnetite, a process known as maghemitization. Magnetite and maghemite are ferrimagnetic, which means that their atoms have opposing magnetic moments. These opposing moments have unequal magnitude, which makes the material magnetic in the absence of an applied magnetic field. Ferrimagnetic materials lose their spontaneous magnetization at their Curie temperature, at which they become paramagnetic (the atoms of the material lose their ordered magnetic moments). Although it is well known that the Curie temperature of magnetite is 853 K, this parameter is difficult to determine in maghemite, since it is metastable and therefore has a high tendency to transform into hematite at temperatures higher than 700 K [115-119].

MNPs for biomedical use are desired to exhibit superparamagnetism. For ferromagnetic and ferrimagnetic materials, when the size of the nanoparticles is below 30 nm, they exhibit superparamagnetic behaviour, which causes their magnetic moments to flip direction under the effect of temperature. The time between two flips corresponds to the Néel relaxation time, and if the particles' magnetization is measured on a much longer time segment than their Néel relaxation time, then their magnetization appears to be zero and the nanoparticles are said to be in the superparamagnetic state. Similarly to paramagnets, superparamagnetic particles can also be magnetized by an external magnetic field, except that their magnetic susceptibility is much higher, since the transition occurs below the Curie temperature of the material [120].

Superparamagnetic iron oxide nanoparticles (SPIONs) are composed of either magnetite or maghemite. Superparamagnetism is the most important factor of the magnetic properties because once the magnetic field is removed, superparamagnetic particles do not retain any magnetism, and consequently the aggregation of the MNPs is avoided in the absence of a magnetic field. A much stronger magnetization is achieved from SPIONs. Although the superparamagnetic behaviour increases with decreasing particle size, particles smaller than 10 nm exhibit a decline in saturation magnetization, thus particle size should be between 10 and 100 nm [113, 121].

5.1.2. Doped Ferrites

Although magnetite is used in all medical applications that include MNPs, there have been new studies on doped ferrites for the same purposes. These include cobalt ferrite ($CoFe_2O_4$) and manganese ferrite ($MnFe_2O_4$). They are not biodegradable and biocompatible like magnetite, and therefore require coatings. $CoFe_2O_4$ has a Curie temperature of 790 K, good mechanical hardness and chemical stability. Its crystalline anisotropy is one order of magnitude larger than magnetite's, which makes it suitable hyperthermia treatment. $MnFe_2O_4$ has a higher magnetic susceptibility than other iron oxides, making it suitable as a contrast agent for magnetic resonance imaging. However, it can also be used for hyperthermia treatment and drug release. Zinc can be added to these oxides to improve their performance. For instance, Mn-$ZnFe_2O_4$ exhibits higher saturation magnetization and therefore has an even better performance as an MRI contrast agent. Furthermore, Co-$ZnFe_2O_4$ has higher magnetic anisotropy which improves its use in hyperthermia [113].

5.2. Synthesis of Magnetic Nanoparticles

The production of MNPs focuses on methods that achieve particles with uniform size and shape. Precipitation from a solution is a technique through which magnetite can be prepared, where an aqueous solution of Fe^{2+} and Fe^{3+} chloride is prepared, to which a base is added. The chemical reaction that defines the precipitation of Fe_3O_4 is given by:

$$Fe^{2+} + 2\,Fe^{3+} + 80\,H^{-} \rightarrow Fe_3O_4 + 4\,H_2O$$

Another technique is co-precipitation, which can be done by two methods. In the first one, ferrous hydroxide suspensions are partially oxidized with different oxidizing agents. For instance, it is possible to obtain spherical magnetite particles with diameters between 30 and 100 nm from a Fe(II) salt, a base and a mild oxidant (nitrate ions). In the second method, mixtures of stoichiometric ferrous and ferric hydroxides are aged in aqueous media, forming spherical magnetite particles with controllable size. In this method, it is possible to decrease particle size by increasing the pH and the ionic strength of the precipitation medium [122].

Microemulsions are thermodynamically stable mixtures of oil, water, a surfactant and frequently a co-surfactant. The preparation of microemulsions is an easy process to perform that produces nanoparticles with good dispersion, controlled size and narrow size distribution. Also known as reverse micelle solutions, water-on-oil microemulsions consist of a continuous oil phase where microdroplets of the aqueous phase are trapped within assemblies of surfactant molecules. Particles of a soluble metal reside in these surfactant microcavities that continuously collide, coalesce and break. By dissolving two reactants in two identical microemulsions and mixing them, the continuous collision and separation of the microdroplets will form a precipitate of both reactants together. Finally, the finely dispersed precipitate can be extracted from the surfactant [122, 123].

It is also possible to synthesize MNPs using polyols. A polyol is an organic compound that contains multiple hydroxyl groups. This method consists of having a solution with liquid polyol as the solvent for a metallic precursor and a reducing agent. The solution is stirred and heated, resulting in the reduction of the dissolved metallic salts and the formation of fine metallic particles [122].

5.3. Surface Functionalization of Magnetic Nanoparticles

Although some MNPs have good *in vivo* behaviour, most require a surface modification that will improve some of their properties. MNPs have hydrophobic surfaces and show poor dispersion in water or organic solvents, leading to agglomeration and increase of particle size, risking losing their superparamagnetic properties. This can be avoided with a ligand exchange step, that consists in replacing hydrophobic ligands at the surface for hydrophilic ones, providing colloidal stability. The surface characteristics are decisive not only on the particles' biocompatibility, but also on cell adhesion. There may be a difficulty in grafting the surface of magnetite nanoparticles due to the lack of organic materials. However, a polymeric coating can add hydroxyl groups and solve this problem. Another aspect to bear is the oxidation of particles. Even though iron oxides are less sensitive to oxidation, the existence of free Fe^{2+} in magnetite particles makes them prone to oxidize and convert to hematite, and consequently lose their magnetism. Besides that, all magnetic particles are toxic except for iron oxide particles, which means that MNPs composed by pure metals, such as cobalt or nickel, and metal oxides, like cobalt ferrite or manganese ferrite, cannot be used unmodified. Particle coating comes as a solution for these issues [113].

Coated MNPs consist of an iron oxide core often coated with organic materials, although inorganic materials may also be used (e.g., gold, silica, carbon). Polymers are the most studied coatings and both natural and synthetic ones can be used. Natural polymer coatings include dextran and chitosan, while synthetic polymer coatings may be composed of polyethylene glycol (PEG) and poly(lactic-co-glycolic acid) (PLGA), for example. Because polymeric coatings may affect the performance of magnetic nanoparticles, it is necessary to regard some aspects such as the hydrophobicity/hydrophilicity, biodegradation characteristics or the molecular weight of the polymer. The thickness of the coating layer should be between 1 and 20 nm [113, 121].

5.4. Magnetic Scaffolds

Magnetic scaffolds have been the subject of several studies throughout the years, towards their ability to stimulate bone regeneration and accelerating the healing process. Bone growth is enhanced by physical stimulations, which is the case of tensile and compressive stresses originated by the body's locomotion. Magnetic scaffolds can convert an external magnetic signal into mechanical stimulus, contributing to cells' activity. The generated magnetic force induces stress on cells and results in changes in gene expression. Mechanoreceptors sense physical forces and lead cells to convert them into biochemical signals through a process known as mechanotransduction, in which

the signals are propagated in order to activate transcription factors that lead to the expression of specific genes. Mechanosensitive ion channels transmit signals to mesenchymal stem cells that may lead to differentiation pathways for an osteogenic lineage and for specific proteins related to bone growth. The study of Xia et al. [124] showed that the differentiation of mesenchymal stem cells into osteoblasts led by the activation of the integrin signalling pathways resulted in the expression of the osteogenic growth factor BMP-2. MNPs' magnetic properties work both in the presence and in the absence of an external magnetic field. Each magnetic nanoparticle can be considered a single magnetic domain, creating nanoscale magnetic fields that can activate sensitive receptors on the surface of cells, enhancing their activity and thus promoting bone regeneration. Moreover, the presence of IONPs in biomaterials increases surface roughness and improves the interactions between the scaffold and cell surface receptors at the cell membrane, since they improve hydrophilicity [103, 112, 124, 125].

Magnetic scaffolds can be obtained by adding MNPs to pre-fabricated scaffolds or by incorporating the MNPs during the production of the scaffolds. The first approach consists of simple physical adsorption. It can be done by dispersing MNPs in a ferrofluid and filling the scaffold material's surface defects and pores by capillarity, for example in ϵ-poly caprolactone or hydroxyapatite. Bock et al. [126] dip-coated hydroxyapatite/collagen scaffolds in aqueous ferrofluids containing IONPs, making the scaffolds magnetic. The second strategy involves blending the magnetic particles with other components of the scaffold during its fabrication. This can be carried out with almost every scaffold preparation technique. For instance, via electrospinning, it is possible to perform the polymerization of a polymer in the presence of magnetic nanoparticles. Recent studies employed other techniques, like Bhowmick et al. [127], who produced chitosan/polyethylene glycol/nano-hydroxyapatite/Fe_3O_4 porous scaffolds via solvent evaporation. Another example was performed by Aliramaji's team [128], that used the freeze-drying method to produce silk fibroin/chitosan/Fe_3O_4 scaffolds. A different study [129] used rapid prototyping to prepare Fe_2O_3/hydroxyapatite/PLLA scaffolds [103, 112].

The efficacy of MNPs incorporation in scaffolds has been proved in numerous studies. Singh et al. [130] produced PCL scaffolds with and without incorporated IONPs. The magnetic scaffolds exhibited enhanced mechanical properties (tensile strength, yield strength, Young's modulus) compared to the non-magnetic ones. The scaffolds were tested *in vitro* with simulated body fluid, where the apatite forming ability was improved by the addition of MNPs. Furthermore, the magnetic scaffolds showed increased alkaline phosphatase activity and expression of genes for type I collagen, osteopontin and bone sialoprotein.

Wu et al. [131] produced ceramic scaffolds composed of hydroxyapatite/β-TCP with and without superparamagnetic nanoparticles, and cultured them *in vitro* with MG63 cells. They observed that the magnetic scaffolds showed good biocompatibility and promoted cell proliferation and differentiation more significantly than non-magnetic samples. Moreover, they implanted the scaffolds in fasciae of rats for 30 days, showing that the expression of BMP-2 was accelerated by the presence of the MNPs, along with new bone formation. Wei's research [132], using Fe_3O_4/chitosan/poly(vinyl alcohol) composite scaffolds on MG63 human osteoblast-like cells has found that the MNPs exhibited ferrimagnetism and biocompatibility, and that they facilitated osteogenesis.

5.4.1. Magnetic Scaffolds Exposed to Magnetic Fields

Although the role of MNPs alone on bone cells viability has been increasingly studied, the application of an external magnetic field allows synergic improved stimulation of bone forming cells. Magnetic fields can be static (SMF), pulsed electromagnetic (PEMF), rotating (RMF) and alternating (AMF). Bone tissue engineering research has focused mostly on SMFs and PEMFs. SMFs are appropriate for long-term bone healing, since they can be induced using a permanent magnet without the need for a power source. According to their intensity, they may be divided in ultra-weak (5 μT – 1 mT), weak (1 mT), moderate (1 mT – 1 T), strong (1 – 5 T) and ultra-strong (>5 T), being moderate intensity the most studied. SMFs have been proven to accelerate the proliferation, migration and differentiation of osteoblast-like cells. Numerous studies have been performed in this regard, both *in vitro*, including in mouse calvarial osteoblasts MC3T3-E1 [133] and human adipose-derived mesenchymal stem cells [134], and *in vivo*, as for example, in rat femurs [135] and rat lumbar vertebrae [136]. The reason for their influence on cells is believed to be the fact that cell membrane is diamagnetic, which means it is repelled by magnetic fields, and therefore the membrane flux is affected by SMFs. However, the exact mechanisms that explain the effect of SMFs on cells remain to be determined. PEMFs have also been shown to enhance osteogenesis and increase bone mineral density. Azadian et al. [137] found that the relevant range of PEMFs was below a frequency of 75 Hz, and that the osteogenic effects were the most obvious between 15 Hz and 35 Hz. Another study by Yan et al. [138] exposed rat calvarial osteoblasts to 50 Hz and a range of 0,6 to 3,6 mT, for 90 minutes a day, and observed that the proliferation and differentiation of the cells was improved by the magnetic fields, and that those exposed to 0,6 mT showed the best results. PEMFs create magnetic fields and electric currents, and the induction of pulsed electric currents in bones produces a sequence of

biological events. However, similarly to SMFs, the mechanisms that justify the benefits of PEMFs or cells are not yet clear [124].

Other studies focusing on magnetic scaffolds under the influence of external magnetic fields have proven an enhancement in osteoblast differentiation. This was the case of Yun et al. [139], that used static magnetic fields with iron nanoparticles to promote formation of new bone and found an increase of alkaline phosphatase activity. Yamamoto et al. [140] tested this in rat calvaria cell cultures with static magnetic fields of 160, 280 and 340 mT during 20 days and concluded that the enhancement of bone formation was exhibited by the increase of calcium, osteocalcin and alkaline phosphatase content. A study performed in 2012 by Panseri et al. [141] developed scaffolds containing hydroxyapatite and magnetite in three different proportions (hydroxyapatite/magnetite ratios of 95/5, 90/10 and 50/50) and tested them in human osteoblast-like cell cultures, under the effect of a magnetic field of 320 mT. The conclusion was that all three proportions showed high biocompatibility with no negative effects arising from the presence of magnetite, and that the cells tested within the scaffolds made of 90% hydroxyapatite and 10% magnetite showed proliferation enhancement at the early stage. Furthermore, the scaffold was tested *in vivo* in a lesion of a rabbit condyle and exhibited a good level of histocompatibility.

In vitro experiments of Ba et al. in 2011 [133], Kim et al. in 2015 [142] and Huang et al. 2017 [129] used magnetic fields of 150 mT, 15 mT and 100 mT, respectively. All of them concluded that weak static magnetic fields promote the proliferation, orientation and migration of osteoblast-like cells and induce osteogenic differentiation in mesenchymal stem cells deriving from bone marrow. Zheng's team [143] cultured dental pulp stem cells under a static magnetic field of 1 mT, which resulted in increased cell proliferation and migration, and induced osteogenesis and mineralization in the cultured cells. Meng et al. [144, 145] produced γ-Fe_2O_3/HA/PLA composite scaffolds using the electrospinning technique and tested them *in vitro* and *in vivo* under a static magnetic field of 0,9-1,0 mT. In the *in vitro* test, they detected an enhancement of the proliferation, differentiation and secretion of extracellular matrix on cultured MC3T3-E1 cells. For the *in vivo* test, the scaffolds were implanted in lumbar transverse defects in white rabbits, resulting in accelerated new bone formation and remodelling.

5.5. Safety of Magnetic Fields and Nanoparticles

Every organism on Earth is under a geomagnetic field. Furthermore, cells, tissues and organs possess their own magnetic fields. Different magnetic fields interact with each other, which is why it is important to understand the possible health effects of magnetic materials. The exposure of bone injuries to magnetic fields can regulate the activity of cells, and the results become clearer with the increase of the field's intensity. However, beyond a certain value, the effects may decrease, become inhibitory or even be toxic. The range of magnetic fields where significant responses by the biological system are observed is called the "biological window". It has been reported by Tian et al. [146] that 2 hour-long exposure of mice to a SMF of 3,5 – 23,0 T does not cause long-term effects. Reddy et al. [147] observed that an exposure of 8 weeks to PEMFs did not increase genetic toxicity and cytotoxicity significantly. However, Halgamuge's [148] research showed that exposure to weak electromagnetic fields could disrupt the production of melanin, which could have a long-term harmful effect on humans. The available data on the side effects of magnetic fields is insufficient to make a definitive claim on their overall safety. Different types and parameters of magnetic fields obtain different responses from bone cells, which is why further studies on this subject are necessary [104, 111].

The implantation of materials containing magnetic nanoparticles demands the assessment of the particles' toxicity. Toxic effects on cells may consist of impaired mitochondrial activity, membrane leakage or morphological changes, which can affect cell viability and proliferation. The toxicity of MNPs is, first of all, related to the composition of the particles. It is known that certain metals, such as cobalt and nickel, have harmful effects on biological entities, while titanium and iron oxide-based particles are much less damaging to cells. Other factors to consider are particle size, shape and surface coating, since they can influence particle aggregation and coagulation, thus eliciting a toxic response. When evaluating the toxicity of MNPs *in vivo*, not only is it important to investigate a possible immune and inflammatory reaction, but it is also necessary to examine the fate of the particles after they are released by cells. The application of MNPs requires studying the effect of accumulated MNPs in tissues and organs, their degradation and their by-products. It is known that the degree of toxicity varies with the type of cells in question, the type of MNPs and a combination of both. It is not possible to make assumptions on these behaviours, which is why it is required to carry out specific research to evaluate the toxicity of a specific MNP on a particular cell type [111].

Magnetic biomaterials count with the inclusion of magnetic particles. The advantage of such particles being superparamagnetic is that they only exhibit magnetism in the presence of a magnetic field. Nonetheless, ferrimagnetic particles can also be used in scaffolds, both in the presence or in the absence of external magnetic fields, being that bone regeneration presents better results in the presence of an external magnetic field. Besides the effect created by the magnetic fields on cells, the presence of these particles in scaffolds enhances the mechanical resistance of the structures. MNPs can be produced through different methods, and their surface can be functionalized to improve their biocompatibility and hydrophilicity. The mostly applied MNPs are iron oxides, since they have very low toxicity and therefore do not necessarily require surface functionalization. Nonetheless, it is crucial that further studies are performed in order to assess the effects that magnetic fields and magnetic nanoparticles may have in the human body.

Conclusions and Future Outlook

Scaffolds play an essential role in providing the environment for cell adhesion and guiding cell proliferation and differentiation in bone repair. Their biocompatibility is crucial to avoid undesirable responses and enable the integration of the material with host tissues. Nonetheless, they must possess other properties to ensure the success of their implantation for bone regeneration, such as biodegradability, proneness to cell adhesion and mechanical properties identical to those of bone. Numerous biocompatible and bioactive materials have been used to produce porous scaffolds and presented very positive results in terms of cells compatibility and differentiation. Bioceramics and polymers are the most widely studied materials for this purpose, as well as composite structures containing both types of material. Bioceramics are bioactive and osteoconductive, and their similarity to bone tissue makes them excellent materials for scaffolds. Polymers are also biocompatible and degradable. Moreover, their mechanical properties are more adequate than those of ceramics, and they allow more processing options for scaffold production.

The inclusion of magnetism in bone tissue engineering has been subject to several studies using different materials, several preparation techniques, distinct types of nanoparticles, and so on. In addition to *in vitro* studies, *in vivo* research has been widely performed, using different animal models and different types of bones. Results show that MNPs have a positive impact on bone cells proliferation and differentiation. They also improve cell adhesion due to their hydrophilicity and enhance the mechanical properties of the overall structure. However, there are numerous variables associated with bone regeneration in humans that are difficult to control. For instance, regarding animals from the same species, they can exhibit different anatomical, biochemical and gene-expression characteristics. These aspects may turn the interpretation of data for clinical application more complex. Furthermore, bone regeneration is a complex process that involves many components and mechanisms acting in different timings. Magnetic materials involve considering numerous parameters, such as their composition, the shape of the material, their preparation process or the intensity of their magnetic field. It is therefore crucial to fully understand how the interactions between MNPs and cells work, in order to determine the optimal parameters that enable achieving the best possible response from the biological system. Even so, the clinical applications of magnetism in bone regeneration are still at a very early stage. The safety of using MNPs is still a question to be answered, since the information about the effects of exposure to magnetic fields and the presence of remains of MNPs inside the body are not yet clearly determined.

In this regard, future work should focus on ensuring the integration of biomaterials and the avoidance of implant rejection. This will most probably be achieved through surface modifications of the substrate, rather than relying on the properties of unmodified materials. Also, the mechanisms through which magnetism influences cell behaviour need to be thoroughly studied, in order to ascertain all the steps involved in producing a magnetic scaffold and obtain an optimal response from it. Furthermore, long-term *in vivo* studies are required to find the biological windows of different types of magnetic fields and to realise the side effects of the presence of magnetic nanoparticles in the biological system, in order to learn the limitations of magnetic scaffolds and how to take the best advantage of them.

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