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Synthetic and Marine-Derived Porous Bone Graft Substitutes

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Abstract: Bone is a dynamic tissue with the capacity of repair and regeneration in specific conditions. Nevertheless, due to the increased incidence of bone disorders, the need of bone grafts has been growing over the past decades and the development of a bone graft with optimal properties remains a clinical challenge. This review addresses the bone properties (morphology, composition and their repair and regeneration capacity) and then is focused on the potential strategies for bone repair and regeneration. It describes the requirements for designing a suitable scaffold material, types of materials (polymers, ceramics and composites) and techniques to obtain the porous structures (additive manufacturing techniques/robocasting or derived from marine skeletons) for bone tissue engineering applications. The main objective of this review is to gather the knowledge on the materials and methods for the production of scaffolds for bone tissue engineering and highlight that natural materials, namely the marine skeletons represent a promising alternative.

Keywords: Bone tissue engineering; biomaterials; bone scaffolds; additive manufacturing techniques/robocasting; marine-derived biomaterials

1. Introduction

Bone is a vascularized connective tissue responsible for the support and protection to the remaining systems and organs of the body, and serve as storage of ions and bone marrow [1,2] Bone as a dynamic tissue is in a constant remodeling process to adapt to the mechanical demands and to repair small lesions that it may suffer [3]. Nonetheless, in the presence of a pathological fractures or large scale bone defect, the capacity of bone repair and regeneration fails [4].

Nowadays, the increase of life expectancy over the last decades is, consequently, related with the extensively use of medical implants, in which bone substitutes are one of the most used. Indeed, bone is the second most tissue transplanted worldwide, right after blood transfusions. The use of bone grafts for tissue repair and regeneration is not only due to the aging population, but can also due to bone fractures, tumor resection and bone diseases [5]. Autologous grafts are the strategy used in the majority of the cases since they contain all the elements essential for bone regeneration (osteogenic cells, osteoinductive growth factors, and a matrix that support bone adhesion and growth), in addition are collected in the individual, which minimize the risks of infection. However, their availability is limited and there is a risk of donor site morbidity [6,7]. Allografts emerge as an alternative to overcome the drawbacks associated harvesting the autografts, however they are associated with risk of infection and a high non-union rate with host tissue [6,8]. Thus, bone tissue engineering has been proposed as a promising alternative to the current bone grafting approaches. Already before Christ, several metals such as bronze and copper were used for the union fractures. It was this type of thinking that persuaded several heath care professionals to try to introduce foreign materials into the bone tissue to compensate bone defects [9].

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A biomaterial may be defined "as any substance (other than drugs) or a combination of substances of synthetic or natural origin, which may be used for any period of time, as a whole or as part of a system which treats, tissue, organ or function of the organism". The performance and success of biomaterials are dependent on the interactions that occur in their interface with the organism, which, in turn, are intrinsically related to the compositional and morphological properties of the material, the health status and daily activities of the individual in whom the biomaterial is inserted and, of course, biocompatibility of the material, which assumes that the use or implantation of a biomaterial does not cause adverse reactions, toxic or carcinogenic to the individual in which it is applied [10,11].

The biomaterials for bone tissue engineering are mainly divided into two main groups, ceramic, and natural and synthetic polymeric materials. Each of these materials have specific advantages and disadvantages [12]. The close resemblance between the chemical composition of the inorganic part of the bone and hydroxyapatite (HA-Ca10(PO4)6(OH)2, almost bioinert) and tricalcium phosphate (TCP-Ca3(PO4)2, resorbable) stimulated intensive research efforts to develop synthetic calcium phosphates based bone grafts, including pure and biphasic compositions [10,11]. The discovery of the bone bonding ability of bioactive glasses through the formation of a layer of HA when exposed to physiological fluid [13] was an important milestone in the development of synthetic bone grafts. Nevertheless, these materials are brittle and exhibited an unprodictable dissolution rate [14]. On the other hand, synthetic polymers have been developed with tailored characterisctics, however due to its lower bioactivity, they can be associated with a risk of rejection. Natural polymers, despite being biocompatible and enhance cell adhesion and differentiation, they have a limited supply and exhibit poor mechanical properties and immunogenicity [15]. Bone is a composite material with an inorganic phase, HA, and an organic phase, mainly composed of collagen. Thus, cceramic-polymer composites stretagy have been widely explored as it combines the two different materials and its drawbacks can be overcome [14–18].

Bone tissue engineering strategie involves the use of porous three-dimensional (3D) scaffold that act as a temporary support and provides a suitable environment and architecture for bone regeneration and development [19]. Scaffolds for bone tissue engineering should have an interconnected porous structure and be highly porous with an adequate size allowing cell adhesion and proliferation and also ensure the diffusion of oxygen and nutrients to the cells and the removal of the waste products. Moreover, the degradation rate should be similar to the formation of new tissue and the mechanical properties should match with the ones of bone [12]. With the conventional techniques used to obtain porous scaffolds is difficult to have control over the pore size, geometry, spatial distribution, and interconnectivity. These obstacles can be overcome using solid freeform fabrication techniques like robocasting in which is possible to have a precise control over the internal scaffold structure [20]. On the other hand, natural marine materials, like corals or cuttlefish bone (CB), due to their unique archinecture have been studied as potential materials to support bone growth [21,22].

2. Bone Tissue

Bone is a specialized, mineralized and vascularized connective tissue that along with cartilage forms the skeletal system. Physically, bone acts as a support and site of muscle attachment for locomotion. The bone rigidity and hardness originated by the deposition of minerals such as calcium phosphate and carbonate inside the organic matrix in an organized functional way allows the skeleton to maintain the shape of the body and the protection of vital organs and bone marrow. In addition, the bone physiological functions include hematopoiesis, a process by which blood cells are formed, and mineral homeostasis, a reservoir for calcium, phosphate, sodium, potassium, zinc and magnesium [1,23].

2.1. Bone morphology

Bone tissue is composed of two main parts, a compact shell called cortical bone and a porous core known as cancellous bone [2]. Cortical bone forms the outer wall of all bones and, in the adult

human skeleton, represents 80% of the skeletal mass. It is composed of repeating osteons units and, in its majority, it is calcified (80% to 90% of volume). It is associated with mechanical properties and, thereby, responsible for the supportive and protective function of the skeleton. The remaining 20% of the bone is cancellous bone, made of an interconnecting framework of trabeculae where only 15% to 25% of the volume of cancellous bone is calcified and in which the empty spaces that are usually filled with bone marrow, the source of undifferentiated cells. It is mainly associated with the metabolic functions but also plays a role in biomechanical functions [1,2,23]. The degree of porosity of cancellous bone contrasts with the denser structure of cortical bone irrigated by a series of fine channels filled by blood vessels. The outer and inner bone surfaces are covered by the periosteum and the endosteum membranes, respectively, that play important roles in the nutrition of the bone tissue and supply of osteoprogenitor cells, which divide by mitosis and differentiate into bone forming cells, osteoblasts and osteoclasts for bone formation and repair [2]. Additionally, the trabeculae and osteon units are composed of collagen and calcium phosphate (CaP) crystals. The collagen fibrils include a 67 nm periodicity and 40 nm gaps between collagen molecules. The CaP crystals are embed in these gaps between collagen molecules and increase the rigidity of the bone (Figure 1) [23].

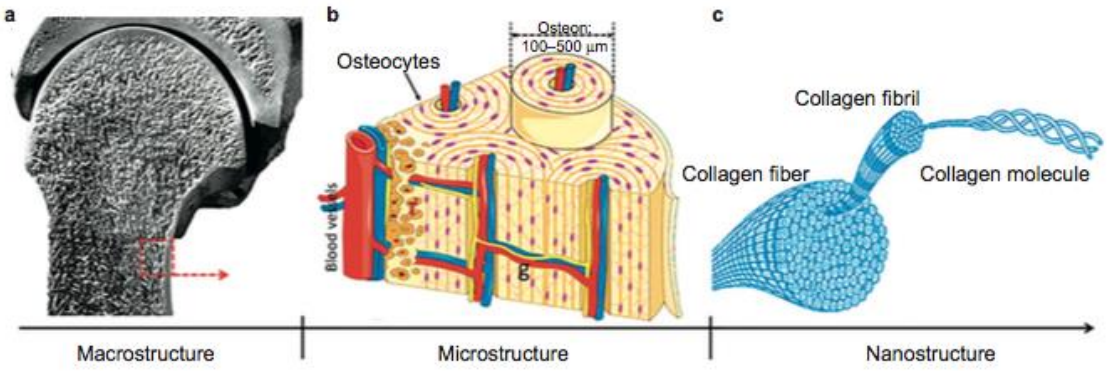

Figure 1. Hierarchical organization of bone tissue: **(a)** Macrostructure of cortical (dense shell) and cancellous bone (porous bone at both ends); **(b)** Microstructures of the osteons (20-30 concentric layers of collagen fibers, called lamellae) and trabeculae; **(c)** Nanostructures of collagen fibrils, which compose the collagen fibers. The HA crystals are embedded in these gaps between collagen molecules and increase the rigidity of the bone [23].

2.2. Bone composition

Bone tissue consists of bone matrix (~ 90%) and bone cell populations (~ 10%), namely osteoblast, osteoclasts, osteocytes and coating cells [2]. The bone matrix is a composite material consisting of 65% mineral phase, HA, and 35% organic phase (~90% type I collagen, ~5% noncollagenous proteins, ~2% lipids by weight) and a residual amount of water [24,25].

2.2.1. Mineral phase of bone

Bone mineral consists of carbonated HA in the form of thin (1,5-4 nm) plate- or needle-shaped, incorporated within collagen fibrils, and orientated with the c-axis in the direction of the fibril [26,27]. The inorganic matrix material consists essentially of a carbonated calcium deficient HA (CDHA) [2]. The carbonate ions (CO32−) might be incorporated into the HA (Ca10(PO4)6(OH)2) lattice partially substituting the OH− sites or the PO43− groups to form A-type HA, or B-type HA, respectively, or AB-type HA when both substitutions occur concomitantly [6]. But several other ionic impurities can be found in the inorganic matrix of biological HA, including small additions of citrate, fluorine, chorine, sodium, potassium, magnesium, strontium, zinc, iron, etc., incorporated in the crystalline lattice or adsorbed to its surface [2].

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2.2.2. Organic phase of bone

Bone mineral consists of carbonated HA in the form of thin (1,5-4 nm) plate- or needle-shaped, incorporatedThe organic phase of bone is mainly composed of type I collagen, nonetheless other proteins, the so-called noncollagenous proteins, accounted ~5% of the total bone weight [28].

Type I collagen is essential in the bone since it provides elasticity to the tissue, stabilize the extracellular matrix (ECM), acting as a template to initial mineral deposition and binds to other macromolecules [28]. This molecule consists in a unique triple helical molecule, forming spaces within the collagen fibrils [29]. Additionally, these spaces are aligned to form thin and extended grooves where the intrafibrillar crystals form and thereby limiting the possible primary growth of mineral crystals, forcing them to be discrete and discontinuous [26,30]. Although type I collagen is the most abundant protein in mature bone, other collagen types, including types III, V and VI are also present in the bone [26].

Besides type I collagen, non-collagenous proteins are also present in the bone. Most of these proteins are not exclusive of bone, however some of them, such as osteocalcin, osteonectin, osteopontin and alkaline phosphatase, play fundamental roles in bone [25,26,28]. Generally, part of these proteins play a structural and mechanical role and other non-collagenous proteins modulate functions of different bone cells by interacting with their cell-surface receptors, proteases, hormones, and other biomolecules including proteoglycans and collagens. Particularly, osteocalcin and osteopontin are important for fracture resistance and in older ostenal bone their concentrations are lower [31]. Additionally, these proteins can also regulate the collagen fibril mineralization and modulate cell division, migration, differentiation and maturation [25].

2.2.3. Bone cells

2.2.3.1. Osteoblasts

Bone mineral consists of carbonated HA in the form of thin (1,5-4 nm) plate- or needle-shaped, incorporatedThe organic phase of bone is mainly composed of type I collagen, nonetheless other proteins, the Osteoblasts, which represent 4-6% of the total bone cells, are cuboidal cells that are accommodated in clusters along the bone surface and, therefore, do not function individually. These cells are derived from osteoprogenitor mesenchymal stem cells of the bone marrow stroma, which are multipotent adult cells that can differentiate into specialized cells including osteoblasts, chondrocytes and adipocytes when appropriate stimuli are applied [32–35]. Osteoblasts are responsible for the synthesis of the bone matrix and subsequent mineralization. This cells are also responsible for the synthesis of organic matrix and regulation of calcium and phosphate fluxes [2]. Under a variety of stimuli, osteoblasts can produce diverse growth factors growth factors including insulin-like growth factors (IGF), plated-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF-β) and bone morphogenic proteins (BMPs) [3,36]. The production of bone matrix by osteoblasts (Figure 2) occurs in three successive phases: production and maturation of osteoid matrix and its subsequently mineralization. Firstly, osteoblasts secrete collagen proteins, mainly type I collagen and non-collagenous proteins and proteoglycan which form the organic matrix. Thereafter, mineralization takes place into vesicular and the fibrillar phases [3,36,37].

2.2.3.2. Osteoclasts

Osteoclasts, responsible for bone resorption, are giant multinucleated cells containing 1 to more than 50 nuclei [1–3,36]. These cells are derived from mononuclear cells of the hematopoietic stem cells lineage, under the influence of a variety of factors [38]. These cells are normally found in contact with a calcified bone surface and within a lacuna (Howship's lacunae) due to its own resorptive activity. Osteoclasts bind to the bone surface through a process that involves binding of integrins expressed in osteoclast with specific amino acid sequences within proteins at the surface of the surface of the bone matrix [3]. Osteoclasts resorb bone (Figure 2) by acidification and proteolysis of the bone matrix and of the HA crystals encapsulated within the sealing zone. Firstly, the process involves the

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mobilization of the HA crystals through the digestion of their link to collagen. Afterwards, the residual collagen fibers are digested and the residues are either internalized or transported across the cells and released at the basolateral domain [3,39].

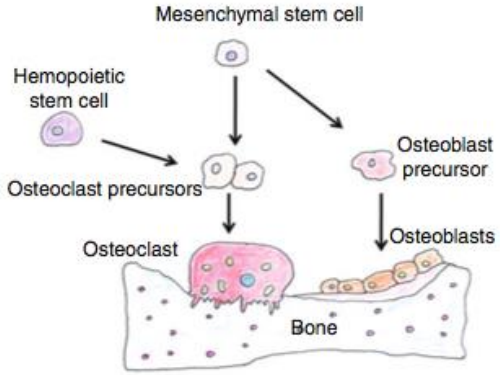

Figure 2. Evolution of osteoblasts and osteoclasts of bone [40].

2.2.3.3. Osteocytes

Osteoblasts have the capacity to secrete bone matrix, during the differentiation process and part of them are immobilized and involved in their own bone matrix and give rise to osteocytes (Figure 3) [40,41]. These are the most abundant cells in bone, representing 90-95% of the total bone cells [36]. These cells are responsible for the detection of microfractures and, thereby, play a crucial role in bone remodelling through regulation of osteoclast and osteoblast activity and function as an endocrine cell [41]. Nevertheless, the functional activity of the osteocytes modifies along the cell age. On one hand a young osteocyte has most of structural characteristics of the osteoblast and a low cell volume and capacity of protein synthesis, on the other hand, an older osteocyte, which is located deeper within the calcified bone, presents with a further decrease in cell volume and an accumulation of glycogen in the cytoplasm. Lastly, the osteocytes are phagocytosed and digested during osteoclastic bone resorption [41].

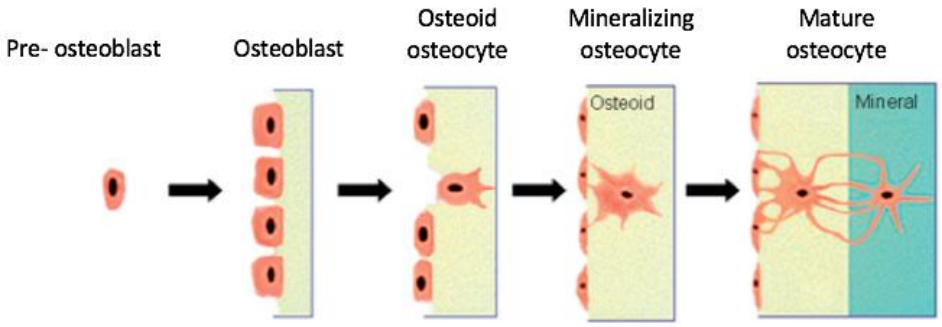

Figure 3. Evolution of bone cells form pre-osteoblast to mature osteocytes. Adapted from [41]

2.2.3.4. Bone lining cells

Bone lining cells are flattened osteoblasts derived from bone matrix and their function is to control the flow of minerals between the bone tissue and the extracellular fluid and the coordination between bone resorption and formation [42].

2.3. Bone remodeling

Bone is a dynamic tissue that undergoes a constant remodelling through life. It possesses a unique self-regeneration capacity, which, in many cases, enables bone injuries and fractures heal without scar formation by responding to metabolic needs and adapting to the mechanical stresses

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applied to the tissue. This process is fundamental to maintain an adequate bone mass, appropriate mechanical properties, and the integrity of the skeleton [3,42–44].

The remodelling is a consequence of a synchronized action of osteoclasts and osteoblast cells. The cellular activity at the remodelling site is characterized by four consecutives phases that transform a resting surface into a remodelling zone: activation, resorption, reversal and formation (Figure 4). The activation phase consists in the recognition of the lesion suffered or the mechanical requests applied by the osteocytes. This activation will cause the retraction of bone lining cells and the recruitment of osteoclast cells through the release of cytokines [32]. Resorption begins upon a signal that leads to the migration of partly differentiated mononuclear preosteoclasts to the bone surface, which consequently form multinucleated osteoclasts. This process is characterized by a demineralization of the bone matrix, from a process of acidification of the zone to be adsorb, dissolving crystals of HA, and degradation of the organic part of the bone by the action of proteolytic enzymes, leading to the formation of gaps. After resorption phase, the reversal phase takes place. During this phase, mononuclear cells prepare the bone surface for bone formation and provide signals for the recruitment of osteoblast precursor cells that will proliferate and differentiate. Once completed the reversal phase, the bone formation takes place where osteoblasts lay down until the resorbed bone in fully replaced by new bone [3,32,42].

The formation of bone tissue occurs in two stages: bone matrix production and its mineralization. Initially, osteoblasts synthesize the osteoid that will function as a support for the deposition of the mineral phase [2]. Mineral deposition occurs between the collagen fibers, due to the conformation of the collagen molecule that acts as a nucleation agent for HA that precipitates as mineral nodules [45]. When bone formation is completed, the osteoblasts can undergo apoptosis or terminal differentiation in osteocytes or bone lining cells, concluding the remodelling process [42]. A prolonged resting period begins until the beginning of a new remodelling cycle [3].

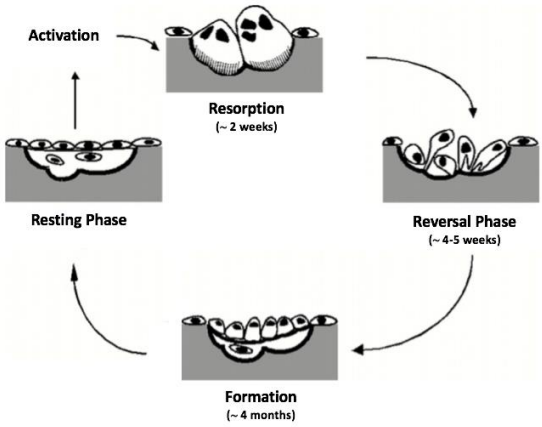

Figure 4. Bone remodelling cycle. Adapted from [1].

Importantly, in a homeostatic equilibrium bone resorption and bone formation take place in a coordinated way and, therefore, old bone is replaced by new tissue adapting to the mechanical load and strain. This homeostatic equilibrium only occurs during the first three decades. Indeed, it is precisely in the third decade when bone mass is at the maximum, and this is maintained with small variations until the age of 50. Posteriorly, resorption predominates and bone mass start to decrease [3,40,43].

3. Bone grafts

3.1. Evolution of life expectancy and the need of bone grafts

The human life expectancy at birth remarkably increased especially since the mid-1800s and continued during the following century [46]. There has been an impressing gain of about 30 years in

life expectancy in western Europe, the USA, Canada, Australia, and New Zealand – and even larger gains in Japan and some western European countries, such as Spain and Italy. This trend is consensually attributed to a complex interplay of advances in medicine and public health coupled with new organization modes at different societal levels, including economic, political and behavioral related changes [47]. Indeed, improvements in medicine are often pointed out as the most impacting factor responsible for the gains in human life expectancy [48]. However, it is worthy to mention that the earlier industrial revolution and the innovations in agricultural production and distribution enabled nutritional diversity and consistency to large numbers of people and are also seen as other important driving factors for lowering mortality [45]. In this regard, the former infectious leading causes of death (infectious and parasitic diseases) were gradually replaced by degenerative diseases such as cancers and diseases of the circulatory system, decreasing dramatically the risk of dying at earlier ages and postponing death to old ages [49].

The fantastic improvements in human life expectancy, the fantastic improvements in human life expectancy, the behavioural nutrition and physical activity, came together with serious consequences in the function of the entire human musculoskeletal system including: (i) the loss of bone mass or density due to decreasing contents of calcium and other minerals by osteoporosis, which result in increased brittleness and breakage of bone. This problem is commonly observed with a higher incidence in women after menopause; (ii) increased incidence of degenerative diseases such as osteoarthritis in which the joints become stiffer and less flexible and the cartilage may begin to rub together and wear away concomitantly with a decrease of the synovial fluid in the joins and the eventually deposition of minerals (calcification). This bone joints breakdown may also lead to inflammation, pain, stiffness, and deformity. There are also an increased incidence of bone tumor resections; (iii) the decrease of the cushioning effect exerted by the gel-like invertebral disks in spine because they gradually lose fluid and become thinner and resulting in shrinkage of the trunk and in a consequently overall height decrease; (iv) increased rateof teeth loss or extraction and the need to maintain dense and healthy jaw through bone augmentation to prevent its natural deterioration; (v) increased incidence of serious trauma injuries associated with lifestyle changes (participation in sports); (vi) the refusal of the public to tolerate the slightest limitation in mobility.

Under these pathological conditions, the potential of bone repair and regeneration fails. In this regard, insufficient blood supply, infection of bone or surrounding tissue, and systemic disease can result in delayed unions or non-unions which, consequently, requires grafts substitutes. When accepted by the body, the bone graft provides a framework for growth of new living bone. As native bone grows, it gradually replaces the graft material resulting in a fully integrated region of new bone.

3.2. Ideal bone graft

An ideal bone graft should provide osteointegration, osteconduction, osteoinduction and osteogenesis [50]. Osteointegration is the ability to have a structural and functional connection between the living bone and the surface of the graft, in this way, not having the formation of fibrous tissue at the bone-implant interface [50–52]. Osteoconduction is a characteristic by which bone grows on the surface of the graft [50,51]. Osteoinduction, is the ability of primitive, undifferentiated and pluripotent cells from the surrounding host tissue to develop osteoprogenitor cells followed by the production of osteoblasts [50,51,53]. Osteogenesis is the capacity to produce new bone by osteoblasts present within the bone graft [50,53].

The most common sources of bone grafts include autografts, allografts, xenografts and natural or synthetic bone grafts substitutes [54]. These different bone grafts and their respective virtues and disadvantages are described in the following section.

3.3. Autografts

Autografts remain as the gold standard constituting approximately 58% of the bone grafts [5,53,55]. These bone substitutes can be harvested from non-essential bone of the own patient such as the iliac crest (the most common source), fibula or metaphyses of long bones. Autografts for dental procedures are typically harvested from the jaw, hard palate, or the chin. If there is not enough bone

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available in these areas, the tissue graft may be taken from the hip or shinbone [7,55,56]. Autografts are known to be an optimal bone graft since it has osteoinductive, osteogenic and osteoconductive properties. Fresh autografts contain all the elements essential for bone regeneration like the preservation the trabecular architecture, the presence of viable cells and growth factors (BMP -2 and -7, FGF, IGF and PDGF). In this way, autografts are rapidly incorporated into the host site with lack immunogenicity [7,53,54]. The periosteum and nutrient artery are generally harvested with the piece of autologous bone and blood vessels that can be anastomosed to the blood vessel at recipient site. Once the transplanted bone is secured into its new location it generally restores blood supply to the bone in which it has been attached. Despite the advantages, the use of autografts implies additional surgery with a donor-site morbidity related to blood loss, wound complications, local sensory loss and chronic pain [50,53]. Additionally, autografts are limited available, required additional surgical time and costs and, therefore, other bone graft options should be considered [50,53–55].

3.4. Allografts

The drawbacks associated with the use of autografts can be overcome by the use of allografts, which in turn represent about 34% of the bone substitutes [55]. Allografts, are derived from human, like autografts, however they are harvested from an individual other than the one receiving the graft [53]. Different sources can be used, they can be harvested from cadavers of human individual donors who donate their bodies for the benefit of science or from a multi-organ donor is another source of bone, which is associated with the long bones acquired under sterile condition in the operating theatre after organ explantation and also from people who are in need of repairing and regenerating bone defects and in which the most common source is the femoral head of a patient undergoing a hip replacement. Before the use of any allograft, the donor must be thoroughly screened to ensure that no infectious diseases are present. Nevertheless there is always a risk of transmission of an undetected viral or bacterial disease [8,57]. Allografts can be applied as structural forms or as bone chips. Additionally, they can be processed as mineralized or demineralized, fresh, fresh-frozed, or fresh-dried forms. Allografts have osteoinductive and osteoconductive properties, however lower osteogenic potential due to the absence of viable cells [8,53,54]. Complications linked with allografts include infections, high non-union rate with host tissue and fracture [50,53].

3.5. Xenografts

Xenografts are another alternative to autografts. This type of bone graft is harvested from nonhuman species, commonly from pigs, cows and horses. The first bone graft procedure dated from 1668 was allegedly performed by the Dutch surgeon Job Van Meekeren graft who harvested a bone derived from a dog's cranium and implanted it in a soldier's skull to successfully repair a traumatic defect [58]. In this regard, due to the large quantity of donors, xenografts may be more readily available and less expensive. Nevertheless, the potential transmission of bioactive material that cause diseases or rejection in the host remains as a threat. Many studies have attempted to investigate protocols that might be suitable to eliminate bioactive components such as heterologous cells, xeno-antigens and DNA material while preserving those that are essential for the proper functioning of ECM such as collagen helical macromolecules and water absorbing proteoglycans [59,60]. The adverse consequences of the decellularization process on the natural organization and physiological functionality of tissues intended as bio-inspired scaffolds were addressed by several authors [61–65] In order to avoid unfavourable immune responses xenografts requires a more sterile process, which can result in a loss of osteogenic and partly of osteoinductive properties [53,66]. An alternative approach to prepare safe xenografts for bone regeneration involves chemical and thermal treatments in order to remove all the organic substances from fresh animal bones. Sang-Hoon Rhee, et al [67] patented a method for preparing safe bovine derived hone graft substitutes, which do not have the risk of infection with bovine spongiform encephalopathy. The method comprises treating bovine bone with sodium hypochlorite followed by a heat treatment at temperatures within the range of 600-1000 °C. The organic substances are burned out and the resulting bone graft substitute does not cause an immune response. The patent is associated to a commercial product (Geistlich Bio-Oss®), which

has allegedly been documented in more than 900 publications [68,69]. Other similar porcine- and bovine-derived commercial bone graft materials are available. Some examples are the products under the trade names of Symbios®, Endobone®, and Straumann® XenoGraft [70].

Even though, several concerns still persist toward the use of bone grafts to face all the health problems derived from aging, trauma and degenerative diseases, leaving room for an increasing search for alternative synthetic bone substitutes [71].

4. Bone Tissue Engineering

The shortcomings of bone grafts and the need to face all the health problems originating from aging, trauma and degenerative diseases constituted the main driving forces for developing new synthetic biomaterials. Other stimulating reasons for the recent boom in the development of new synthetic biomaterials and implantation devices include: (i) increasing awareness among patients and doctors of the numerous co-morbidities associated with autograft harvesting; (ii) the elevated regulatory scrutiny and recalls imposed on allograft tissue banks for distributing human bone and soft tissue products that were improperly screened for infectious diseases; (iii) The recent developments in surgical procedures and materials that allow new procedures to be available. Accordingly, a wide variety of synthetic bone substitutes have been developed and employed over the past 50 years, contributing to the actual market trend for shifting away from autografts to bone graft substitutes, and from cadaveric allografts to synthetics [70].

Bone tissue engineering is a multidisciplinary field that applies the knowledge of bioengineering, biology, cell transplantation, and material science to create new biomaterials that will interact with biological systems to treat, strengthen and, thereby, regenerate damage tissues and restore their function, instead of replacing them [12,72]. Normally it involves the use of porous three-dimensional (3D) scaffolds that along with cells and bioactive factors provide structural support for cells to spread, migrate, multiply and differentiate and new tissue being formed [12,73]. In this way, scaffolds act as a temporary ECM inducing the natural processes of tissue regeneration and development [74].

4.1. Scaffolds for bone tissue engineering

A wide variety of biomaterials and manufacturing techniques have been explored to produce a scaffold able to regenerate the bone. When designing an ideal scaffold for bone tissue engineering, some important characteristics should be considered: (i) firstly, the scaffold must be biocompatible. They should have the ability to support cell adhesion and proliferation onto the surface and through the scaffold, without any negative effect to the host tissue, which may lead to a reduced healing or cause rejection by the body [75]; (ii) the underlying premise of bone tissue engineering is to allow overtime the replacement of the implanted scaffold by the ECM. In this regard, the scaffolds must be biodegradable and preferably able to degrade at a similar rate to bone formation. The by-products originated from the degradation process should be non-toxic and roved from the body without interference with other organs [12]; (iii) the scaffolds must have a highly interconnected porosity for successful bone growth and vascularization. Nowadays, it is recognized that a hierarchical porosity from macro to nanoporous is beneficial for the development of new bone tissue [55,76]. Scaffolds with a pore size between 200 and 350 μm reveal to be optimal for bone growth. It allows cell infiltration and, subsequently, formation of ECM as well as the diffusion of nutrients and oxygen and the removal of waste products [75]. The presence of micro and nano porosity plays a fundamental role on cell attachment, biomineralization and an in vivo osteointegration [76]; (iv) An ideal bone scaffold should have mechanical properties that match to the host bone properties and should be strong enough to allow surgical handling during implantation. Importantly, bone scaffolds must maintain their integrity from the time of implantation to the end of the remodelling process. The healing rate significantly modifies according with the age, which should be considering when designing the scaffolds. Fractures from young individuals normally heal in approximately six weeks and mechanical integrity returns one year after fracture, however, in elderly people this bone regeneration slows down. Furthermore, it is known that optimal mechanical properties, often

occurs in detriment of high interconnected porous scaffold, which in vitro could have potential, however, when implanted in vivo it fails due to the insufficient capacity for vascularization. With this in mind, a balance between mechanical properties and porous structure is fundamental to design a successful scaffold [12].

4.2. Biomaterials for bone tissue engineering

Biomaterials has evolved through three generation. The first generation of biomaterials was developed during the 1960s and 1970s and intend to provide adequate functional properties without harmful effect on the host. This generation biomaterials were not specifically developed for medical use consist and, therefore, represent widely available industrial materials like elastomeric polymer and silicon rubber. These materials were selected since they have suitable physical properties and were bioinert. With the emergence of the first generation of biomaterials tens of millions of individuals have improved their quality of life in 5 to 25 years. The goal of second generation of biomaterials was to shift from a completely bioinert reaction to the production of materials that could induce a controlled reaction with the host tissue in order to have a therapeutic effect and hence have a bioactive behaviour. This generation of materials are associated with the development of resorbable biomaterials in which the degradation rate could be adjusted to the desired application. In this way, the implanted material could be degraded into soluble, non-toxic product by the host and, consequently, eliminate the interface between the implanted site and the host tissue. These materials are in clinical use in fields like orthopaedic and dental surgeries, in localized drug release applications and in cardiac assist device. The third generation of biomaterials has the objective to support and stimulate the regeneration of functional tissue and, thereby, induce cellular responses at the molecular level by using two routes, tissue engineering and in situ tissue engineering. These approaches involve the use of scaffolds, cells and growth factors and has already been responsible for the successfully replacement of damaged skin, cartilage, bladders, corneal epithelium and trachea [11,77,78]

The field of biomaterials for bone tissue engineering is in constant evolution. These biomaterials can be subdivided into organic polymers, which can be subdivided according to its synthetic or natural source, and inorganic materials such as calcium phosphates and bioactive glasses. By combining the former materials, it is possible to form composites [12,79,80].

4.2.1. Organic materials

Polymer materials are extensively used in tissue engineering. They can be divided into natural and synthetic polymers.

4.2.1.1. Natural polymers

Natural polymers constitute the native ECM and, thereby, have an excellent biocompatibility and have a low immunogenic potential. They are bioactive as they have the capacity to interact with the host's tissue. Its structure is organized and comprise ligands that can be bound to cell receptors. [18,81,82]. Although in some cases (e.g. chitosan and starch) their source is almost unlimited, most of them lack enough quantity. They are difficult to process and its degradation modifies from patient to patient since the degradation depend on enzymatic processes [82].

Collagen is the main component of ECM and, therefore, has an inherent biocompatibility, non-cytotoxicity and non-inflammatory reaction. It has functional groups that improve cell adhesion and proliferation. This natural polymer, despite having lower mechanical, it has a stable structure related to covalent cross-linking among the collagen fibrils. Moreover, it can be processed into different forms, powders, sponge's foams, sheets, fibres, membranes, films and injectable viscous solutions. However, collagen has a variation bath-to-bath in terms of physicochemical and degradation properties and is associated with a relative risk of infection [81,82].

Chitosan, the deacetylated chitin derivative, is a natural polymer with interestingly properties for biomedical applications due to its biocompatibility, biodegradability, low toxicity, non-

immunogenicity, intrinsic anti-bacterial nature. This polymer enables cell adhesion and proliferation and, in addition, support the formation of bone matrix. As collagen, it can be processed into different forms. The degree of degradation can be adjusted regarding the degree of deacetylation [81,82].

Alginate, a polymer obtained from brown algae, has been widely used in the biomedical field. It has an exceptional biocompatibility, biodegradability and non-toxicity. It is not expensive polymer and are available in abundance. Alginate is a polymer that that can be easily modified, and, for instance, alginate gels produced through the cross-linking with calcium, can be introduced through a minimal invasive procedure. Some drawbacks are associated with alginate like the slow degradation rate and inappropriate mechanical integrity that precludes long term biomaterials implants.

Hyaluronic acid, obtained from ECM of all connective tissue, exhibited good biocompatibility, immunoneutrality and viscoelasticity [81,82]. It is beneficial for bone regeneration [83].

4.2.1.2. Synthetic polymers

Synthetic polymers can be obtained under controlled conditions and, thereby, have a predictable batch-to-batch uniformity with reproducible and adjustable physicochemical properties (e.g. mechanical behaviour and degradation rate). It is possible to tailor the shape, porosity and pore size of synthetic polymers and incorporate chemical functional groups that improve tissue growth [81]. Normally, the degradation of synthetic polymers occurs through an hydrolysis process and, in this way, it does not vary between hosts [84].

Polyesters are the most used polymers in the field of bone tissue engineering and includes poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(lactic-co-glycolide (PLGA), which is a copolymer of PGA and PLA, and poly(ε-caprolactone) (PCL). Polyesters can be dissolved in organic solvents, with the exception of PGA, which is only soluble in highly fluorinated solvents due to its highly crystalline structure. These polymers are biocompatible and the by-products originated from degradation are glycolic acid and lactic acid that are natural metabolites and, therefore, not harmful for the human body. Nevertheless, these by-products can reduce the local pH and, consequently, may induce an inflammatory reaction[81,82]. PGA has a short degradation period (from 4 to 12 months) and high values of tensile strength and modulus of elasticity. On the other hand, due to its hydrophobicity, the degradation rate of PLA stands at between 12 months and 2 years. The low values of PLA tensile strength and modulus of elasticity can be improved by the use of copolymers of lactic acid and glycolic acid like PLGA [82,84]. PCL has a degradation rate slower than the other polyesters, it can achieve 24 months. An improvement of this degradation rate can be achieved by a co-polymerization process. Additionally, PCL is biocompatible and exhibit suitable mechanical properties for bone tissue engineering.

Poly(propylene fumarate) (PPF) is an unsaturated linear polyester that upon degradation originate propylene glycol and fumaric acid, products that are biocompatible and easily removed from the body. The mechanical properties can be improved via cross linking through the fumarate double bond or via thermal or photo cross linking through the active carbon chain double and in this case also the degradation rate can be tailored. [81,82]. PPF is in liquid form before cross-linking, which allows it to be injectable and, consequently, being suitable for orthopaedic implants in minimal invasive procedures.

Polyanhydrides are biocompatible degradable polymers with good properties of drug-controlled release. Nevertheless, they lack appropriate mechanical properties for load-bearing application. To overcome this drawback, polyanhydrides are copolymerized with polyamides with surface-eroding properties [81,82] or alternatively be photo cross-linkable and be injectable [82].

4.2.2. Inorganic materials

CaP such as β-tricalcium phosphate (β-TCP) and HA and bioactive glass has been explored as bone substitute biomaterial due to their chemical and structural similarity to the mineral component of bones and teeth.

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4.2.2.1. Calcium phosphates

CaP materials (Table 1) can be found under various forms from thin coatings in metallic implants improving their biocompatibility to temporary structures that are replaced by new bone. They can be produced in large quantities, with a relatively low-cost. In addition, CaP are stable and, therefore available off-the-shelf [27,85].

HA and β-TCP are two of the most used CaPs. HA is one of the most stable phase under physiological conditions and has a low solubility and, consequently, a slower resorption. Traditionally, HA is prepared in aqueous solution by mixing the adequate quantities of Ca- and PO_4-containing solutions at pH above 9, followed by filtration, drying and sintering. The unsintered HA is poorly crystalline and often non-stoichiometric. On the other hand, β-TCP is a high temperature phase, which is only prepared at temperatures above 800°C. However, it is important to notice that approximately above 1125°C β-TCP transforms into a high temperature phase α-TCP. When compared with HA, β-TCP is more soluble and have lower mechanical stability [85,86]. Therefore, an optimum balance of a more stable phase of HA and a more soluble phase of β-TCP, which forms the biphasic calcium phosphates (BCP), leads to a material with a controlled bioactivity and balance between resorption/solubilisation which guarantees the stability of the biomaterials while promoting bone ingrowth [87,88]

Table 1. Main calcium orthophosphate componds [85,89–91].

	Formula	**Ca/P molar ratio**	**Mineral**	**Symbol**
Monocalcium phosphate anhydrous	$Ca(H_2PO_4)_2$	0,50	—	MCPA
Monocalcium phosphate monohydrate	$Ca(H_2PO_4)_2{\cdot}2H_2O$	0,50	—	MCPM
Dicalcium phosphate anhydrous	$CaHPO_4$	1,00	Monetite	DCPA
Dicalcium phosphate dihydrate	$CaHPO_4{\cdot}2H_2O$	1,00	Brushite	DCPD
Octacalcium phosphate	$Ca_8H_2(PO_4)_6{\cdot}5H_2O$	1,33	—	OCP
Precipitated amorphous calcium phosphate	$M_u(Ca_3)(HPO_4)_{3v}(PO_4)_{3y}{\cdot}z2H_2O$)[1]	0,67-1,50	—	ACP
α-Tricalcium phosphate	α-$Ca_3(PO_4)_2$	1,50	—	α-TCP
β-Tricalcium phosphate	β-$Ca_3(PO_4)_2$	1,50	—	β-TCP
Calcium-deficient hydroxyapatite	$Ca_{10-x}(HPO4)_x(PO4)_{6-x}(OH)_{2-x}$[2]	1,5-1,67	—	CDHA
Sintered hydroxyapatite	Ca10(PO4)6(OH)2	1,67	Hydroxyapatite	HA
Tetracalcium phosphate	$Ca_4(PO_4)_2O$	2,00	Hilgenstockite	TTCP

[1] M is usually a monovalent cation (Na+, K+, NH4+) which is present only if there is an overall negative charge on the calcium phosphate. u may vary between 0 and 3, v may vary between 0 and 1,5, y may vary between 0 and 0,667 and z is unclear at this point. The ACP produced under basic conditions has normally u=0, v=0 and y=0,667 and, therefore, the following composition: Ca3(PO4)2.z2H2O where z=3–4,5.

[b] x may vary between 0 and 1. When x=1 (the boundary condition with Ca/P=1,5), CDHA has the following composition: Ca9(HPO4)(PO4)5(OH).

Since CaP are similar to the mineral phase of bone, they are recognized as biocompatible, a material not foreign to the body and also non-toxic. Importantly, CaP exhibit a bioactive behaviour and are integrated into the body by processes equal to those of bone remodelling, leading to an intimate physicochemical bond between the biomaterial and bone. Moreover, CaP are known to osteoconductive and support cell adhesion and proliferation [89]. The main drawback associate with CaP biomaterials is their mechanical properties, namely their brittle nature with a low fracture strength, represent a concern in high load-bearing applications [27,80,85,89]. This brittle nature is associated with the high strength ionic bonds and can be manipulated by composition, crystallinity, grain size and boundaries and porosity [85].

The crystalline structure of CaPs enables the incorporation of trace amounts of certain ions existing in bone composition [92]. It is recognized that the introductions of ions like strontium (Sr) [93–96], magnesium (Mg) [94,96], manganese (Mn) [7,95,96] or zinc [97,98] in a single or combined way play a fundamental role in bone development. Sr is present in bone in considerable amounts and, in particular at regions of elevated metabolic turnover [92,96]. Its presence is associated with the increase of osteoclast apoptosis and the enhance of osteoblastic cell proliferation and collagen synthesis, which subsequently maintain the bone formation and inhibit bone formation [93,94]. Mg is related with mineralization of calcified tissue, its amount starts to be higher and decrease during the calcification process. Further, this ion influence bone metabolism as it plays a role in osteoblast and osteoclast activity [92]. The incorporation of Mn has a positive effect on bone growth as it promotes cell adhesion due to the fact that in the presence of Mn there is an increase of the ligand-binding affinity of integrins, a receptor that mediates the interaction between [96]. Zn in a similar way as Mg promotes bone formation and its deficiency in the body is associated with the decrease of bone density. Furthermore, Zn influences crystallinity and morphology of biological apatite crystals [97,99].

4.2.3. Composite materials

Hard tissues, like bone, should be able to support the load and when compared to soft tissues should be more stiffener and stronger. Thus, in most cases, instead of polymers, ceramics and metals have gained more attention. Nevertheless, some important drawbacks are associated with these two materials. Ceramics are more brittle and, in some cases, stiffer than bone and metals are considerable stiffer than the bone. On the other hand, despite being more ductile, normally polymers do not exhibit enough stiffness for bone grafts applications (figure 5) [100]. With this in mind, composite materials have been widely explored in bone tissue engineering field since it combines two different materials that resulted in an improvement in mechanical properties and osteoconductive properties of bone grafts.

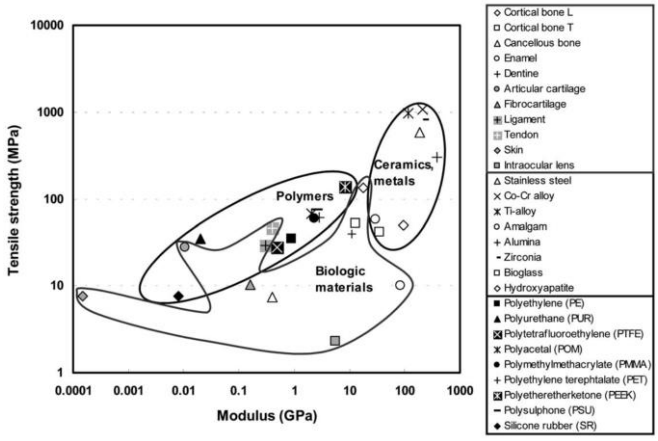

Figure 5. Graphic representation of tensile strength vs. modulus of materials with potential for composite design regarding biomedical applications [100].

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This review will be focused on composites that combine ceramic and polymer materials and despite recognizing that these composite scaffolds can be obtained by mixing ceramic powder with a polymer solution and using different manufacturing techniques [17,101,102], ceramic deposition onto polymers [17,103,104] or by polymer deposition onto ceramics [17,105–109], only the last one will be reviewed.

Motealled et al. [105] produced 45S5 bioglass scaffolds by robocasting and studied the effect of their coating with natural (gelatin, alginate and chitosan) or synthetic (PCL or PLA) polymers on the mechanical and in vitro bioactivity and degradation behaviour. Chitosan coating is highlighted for its mechanical and biological properties. The incorporation of this polymer demonstrated to have the highest compressive strength and strain energy density at 20% strain. An improvement on the bioactivity of the 45S5 scaffolds that accelerated the formation of an apatite layer was registered in the presence of chitosan coating. In addition, in the majority of the coatings, there was a decrease in degradation rate having a positive impact on the evolution of their mechanical properties. Shi et al. [109] produced a β-TCP scaffold through the polymeric sponge replication method and aimed to improve the mechanical properties of the scaffolds by PCL addition. It was observed that the addition of PCL significantly improved the compressive and bending strength and the highest value was registered with the scaffolds containing 40% of β-TCP and 5% of PCL. Furthermore, the presence of PCL did not compromise the osteoblast cells proliferation and differentiation. The scaffold reinforcement with not only PCL but also with HA particles was studied by Roohani-Esfahani et al. [108]. The authors produced BCP scaffolds and, subsequently, coated with PCL and nano (needle shape) or micron HA. The coating enhanced the compressive strength from 0.1 ± 0.05 MPa of BCP scaffolds to 0.29 ± 0.07 MPa and 2.1 ± 0.17 MPa when micro or nano HA were respectively combined with PCL. Moreover, in comparison with the other scaffolds, the one coated with needle shape HA and PCL exhibited the strongest osteoblast differentiation with a higher alkaline phosphatase (ALP) activity and an upregulation of osteogenic gene expression, namely runt-related transcription factor 2 (Runx2), collagen type I, Osteocalcin (OC) and bone sialoprotein. Apart from the former advantages, the polymer coating onto ceramic scaffolds has been also widely used for drug delivery systems. For instance, Li et al. [106] coated 45S5 scaffolds with a solution containing poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and vancomycin, an antibiotic used for infections that occur during bone disease treatment. The authors observed that the polymer coating improved the compressive strength and mechanical stability and, additionally, the in vitro bioactivity had not been negatively influenced by the coating. A sustain and controlled drug release was observed in the coated scaffold (99.9% in 6 days), contrary to what was observed when the drug was directly adsorbed on the 45S5 scaffold (99.5% in 3 days). The thickness and structure of the polymer coating can be dependent on the texture of the scaffold material, in specific on the specific surface area (SSA). Canal et al. [107] studied the influence of the previous parameters on the Simvastatin acid (SVA) release and to that end produced β-TCP and CDHA scaffolds coated with polycaprolactone-co-polyethyleneglycol (PCL-co-PEG) and loaded with SVA using a low pressure plasma process. This coating process allowed the coating of inner regions of the scaffolds up to a certain depth. SVA is of interest in bone healing due to its osteogenic and angiogenic properties. CDHA scaffolds exhibited a SSA 33 times higher than β-TCP scaffolds and, thereby, on the β-TCP scaffolds, the thickness of the drug-loaded polymer layer was three times higher and the drug loading capacity was lower. Regarding the SVA releasing profile, it was observed after 5 h a release of 98% in the uncoated β-TCP, while in the uncoated CDHA, the release occurred for more than 10 days. When coated, a drug diffusion was only registered in the coated CDHA scaffolds, in which the SVA release was blocked in the first 1.5 h and subsequently a slow diffusion was registered along 11 days, the timeframe of the experiment.

5. Scaffold fabrication techniques for bone tissue engineering – Robocasting

As previously mentioned when designing a scaffold for bone tissue engineering several aspects should be taken into consideration, namely the porosity at different dimensions to allow cell adhesion and proliferation, but also vascularization for a subsequent bone growth and vascularization.

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Mechanical properties are also fundamental to provide an adequate mechanical support for bone repair and regeneration. In conventional porous scaffold fabrication techniques (e.g. freeze drying, chemical/gas foaming, melt molding, phase separation, fiber meshing, supercritical-fluid technology, and solvent casting in combination with particulate leaching) it is difficult to have control over the pore size, geometry, spatial distribution, and interconnectivity [20,78,110]. These obstacles can be overcome using additive manufacturing (AM) techniques, which enables the production of scaffolds with a precise control of internal scaffold architecture, without the need for subsequent machining [20,80,110]. AM technologies enables the creation of complex 3D layer-by-layer structures directly from a computer-aided design (CAD) file and data from computerized tomography or magnetic resonance image medical scans can be used to create CAD models that are then converted into STL-files. By using these STL-files it is possible to match the scaffold's external shape to the damage tissue site [20,78,110]. Along with important advantage previously mentioned, AM techniques do not require many process steps and have a small manual interaction [78].

Robocasting is one of AM technique that allows to build scaffolds using a concentrated colloidal suspension (ink/paste) with neglible organic content and without the need for a sacrificial support or material [110,111]. There is a robotic deposition though a nozzle of a continuous filament capable of fully support their own weight during the assembly layer-by-layer. To prevent non-uniform drying during assembly, during the fabrication process the process of deposition is made within a non-wetting oil bath (Figure 6) [110,112,113].

The colloidal inks developed for robocasting requires a careful characterization and must satisfy two important criteria. Firstly, the ink must exhibit a well controlled viscoelastic response so that it yields upon extrusion but set immediately upon deposition to facilitate shape retention. Second, the ink must have high colloidal volume fraction to minimize drying-induced shrinkage after assembly, that is the particle network must be able to resist compressive stress arising from capillary tension and, therefore, the extruded filaments can retain their shape across unsupported spans, and the subsequent layers do not cause the layers beneath to yield and deform significantly. These criteria require a control over the colloidal forces to first generate a high concentrated stable suspension and then induce a system change that promote a fluid-to-gel transition [111,114].

Porosity in robocasting can be tailored on macro- (greater than 100 μm), micro- (1–30 μm), and submicron (less than 1 μm) scales. Macroscale porosity is introduced directly by the robocasting process as it draws successive layers. Robocast filaments arranged in latticed patterns create macroporous pathways in three dimensions. By varying the rod spacing and size, these pathways can be precisely constructed to produce high uniform macropores. By incorporating porogen microspheres into the suspension prior to extrusion, it is possible to create micropores. With a suitable volume fraction of microspheres, the microporosity is interconnected. Lastly, the submicron porosity can be controlled by varying the temperature profile at which the produced scaffolds are sintered [114].

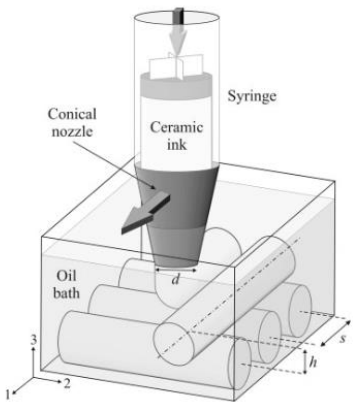

Figure 6. Schematic representation of robocasting fabrication process. The ceramic scaffold is built layer-by-layer from a CAD model. The 3-axis robotic arm moves the injection syringe while

expressing the ceramic ink through the conical deposition nozzle of diameter *d*, to create the desired structure immersed in an oil bath. The layer spacing, *h*, and in-plane rod spacing, *s*, are represented in the scheme [115].

6. Marine-derived bioceramic as scaffolds for bone tissue engineering

Over the last years, the production of scaffolds significantly changes with the introduction of AM techniques and the significant increase of the release of affordable AM bioprinters. Nevertheless, however, a major roadblock identified is the development of technologies that facilitate the implementation of these bioprinters in regenerative medicine clinical manufacturing. One of the most vital, but to date limiting, components required for widespread adoption of bioprinting in regenerative medicine is the availability of effective bioinks. (REF3).

Oceans are abundant sources of diverse materials with potential applications in healthcare, including, among others, bioceramics, biopolymers, fatty acids, toxins and pigments, nanoparticles, and adhesive materials [116]. In this regard, and in order to overcome the drawbacks associated with AM, marine skeletons, mainly composed of aragonite ($CaCO_3$), have proved to be a promising alternative for bone tissue engineering taking advantage of the porous structure and mechanical strength [21,117].

Once cleaned, marine skeletons can be used as bone grafts substitutes, either in aragonite or preferably after being hydrothermally transformed into calcium phosphate scaffolds, while keeping exactly the same porous architecture. The transformation can be partial or total, depending on the hydrothermal treatment conditions (temperature, time, chemical environment). The partial conversion of $CaCO_3$ from marine exoskeletons into calcium phosphates mean that the obtained products consist of composite materials with an inner calcium carbonate core and an outer layer with a composition close to that of mineral part of the bone, turning them viable bone grafts substitute materials [22,118–121]. The conversion from corals to porous HA was first performed by Roy and Linnehan in 1974 [118]. Since then, marine skeletons of cuttlefish [122], marine sponges skeletons [123] and nacre seashell [124] have been converted to HA, while maintaining their original structures, aiming at obtaining bone graft substitutes. In turn, sea urchin spines that consist of large crystals of Mg-rich calcite [$(Ca,Mg)CO_3$] have been hydrothermally transfored into Mg-substituted TCP [125] has been used as templates with optimal range of pore size, channels, and structural network for bone growth.

The following sections of. This review will be focused on the use of corals and CB as scaffolding systems in the area of biomedical applications.

6.1. Corals

Corals are marine invertebrates typically living in compact colonies of many identical individual polyps. Each polyp is a small sac-like animal with only a few millimetres in diameter and a few centimetres in length and has a set of tentacles surround a central mouth opening. Near the base, polyps absorb elements present in seawater, namely carbonic acid and calcium ions and produce a calcium carbonate exoskeleton in the aragonite form, which grows over many generations. Apart from calcium carbonate, which represents 97-99%, corals also have oligoelements (0.5-1%), sodium (0.4-0.5%), magnesium (0.05-0.2%), amino acids (0.07%) and potassium (0.02-0.03%). Individual heads may grow by asexual reproduction of polyps. But polyps also breed sexually by releasing gametes simultaneously over a period of one to several nights around a full moon [121,126]. Polyps feed on a variety of small organisms, from microscopic zooplankton to small fish. These organisms are immobilized or killed by the poison carried in the nematocysts existing in the polyp's tentacles, which is discharged in response to a contact with another organism. The tentacles then manoeuvre the prey to the mouth and then into the stomach [127].

6.1.1. Coral-derived bone grafts substitutes

Coral derived bone graft substitutes have attracted the interest of many experimental researches aiming at the characterization, selection of the most appropriate coral species for the intended

applications, and at evaluating their in vitro and in vivo performances. The interest in this topic is also highlighted in a few review articles giving account of the literature reports published mostly along the last three decades [121,128,129]. It has been explored the use of only natural or HA-derived corals but also the combination of these porous structures with cells and/or growth factors. The various coral bone graft substitutes currently available for experimental and biomedical applications and ongoing investigations of coral derived bone replacement materials are summarised elsewhere [129]. Porites, Goniopora, and Montipora digitate (Figure 7), also known as finger coral have been some of the most common coral species exploited in medical applications [129–131]. Porites species possess anatomical structure, physical, and chemical characteristics that simulate more closely the cortical bone, with a porosity <60%, and interconnecting pore sizes of ~190 ◎m, the average diameter of an osteon in human bone. The structure of Goniopora resembles more that of cancellous bone with a porosity >70% and larger pore sizes [129,130].

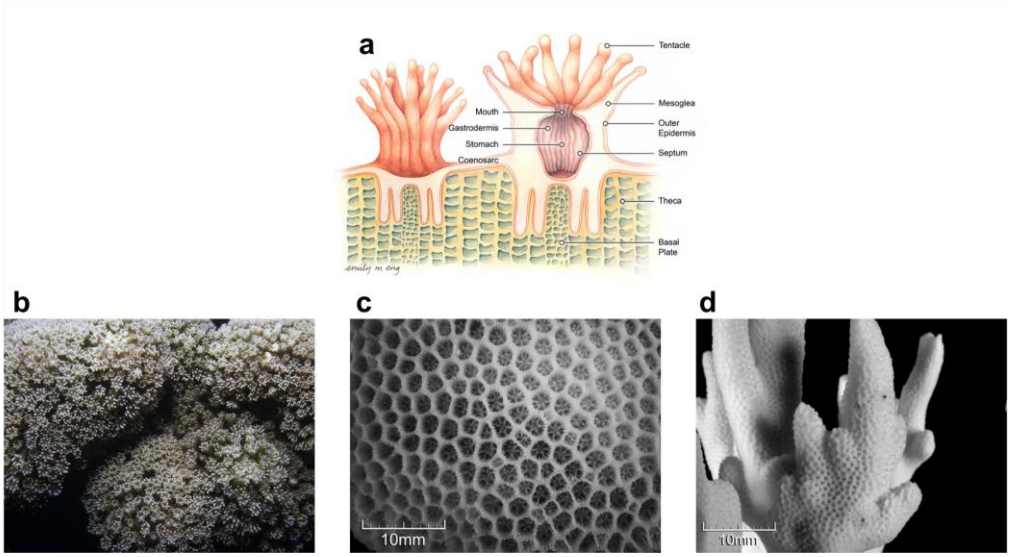

Figure 7. Corals: **(a)** Cross-section of a coral polyp. Illustration credit: Emily M. Eng; **(b)** Porites coral. Image credit: Martha Holmes, Nature Picture Library; **(c)** Goniopora coral. Image credit: Australian Institute of Marine Science; **(d)** Montipora coral. Image credit: Australian Institute of Marine Science.

6.1.1.1 Natural and partial transformed corals

Sergeeva et al. [132] studied the cytocompatibility and biocompatibility of 5 coral scaffolds derived from Acroporidae and Pocilloporidae. Cytocompatibility was in vitro evaluated using human fibroblast and by the formazan assay (MTT) and their biocompatibility was in vivo studied by implantation of the scaffolds into bone defects in rats. All of the specimens were cytocompatible and biocompatible [132]. A comparison between coral and autograft was accomplished by Puvanesway et al. [133] with the aim to study their morphological and chemical composition as well as the osteogenic differentiation potential in vitro using rabbit mesenchymal stem cells (MSCs). The SEM analysis of bone and coral grafts revealed interconnected pores, and micro-CT measurements confirmed pore sizes in the range of 107–315 μm and 103–514 μm, respectively, with a total porosity fractions >92%, what seems to be exaggerated in comparison to other reported values [129]. Significantly higher levels of osteogenic differentiation markers, namely, ALP, OC, and of Osteonectin and Runx2, Integrin gene expression were detected in the coral graft cultures in comparison with those in the bone graft cultures. The authors concluded coral graft enhanced osteogenic differentiation of rabbit MSCs relatively to bone graft culture system.

Mangano et al. [134] used calcium carbonate in sinus elevation procedures and evaluated its clinical performance through histologic and histomorphometric analysis. After a 6-month post

implantation, the mean vertical bone gain was about 7 mm and the histomorphometric analysis revealed a residual calcium carbonate of ~15%, ~28% of newly formed bone, and ~57% of marrow spaces. The reported implant survival rate after 1 to 5 years of follow-up was 98.5% [134]. The osteoconductivity and suitability of coral-derived calcium carbonate was compared with S53P4 bioactive glass and allogeneic fresh frozen bone by Gunn et al. [135] through the implantation of the material into cylindrical bone defects drilled in the femoral condyles of adult rabbits. Histologic and histomorphometric analyses were performed at 3, 6, 12 and 24 weeks. All three materials were found to be biocompatible and osteoconductive. Coral was observed to degrade more quickly, leaving more empty space in the defects, being considered the least suitable bone filler, with no statistically significant difference being observed between the allograft and the bioactive glass [135].

As previously mention, calcium carbonated coral skeletons can be hydrothermally converted into calcium phosphate scaffolds. In this regard and in order to understand whether coral-HA can be a promising alternative to intraarticular autologous structural bone graft, Koëter et al. [136] fill a defect in the femoral trochlea of goats with coral-HA scaffold. They showed that coral-HA did not cause an inflammation reaction and there was a good bone growth in the defected filled with the scaffolds [136].

The potential of coral-HA along with other biomaterials such as cryopreserved bone allograft (CBA), demineralized freeze-dried dentin (DFDD) and cementum for periodontal regeneration was studied by Devecioğlu et al. [137]. The authors studied in vitro the adhesion, proliferation and mineralization of periodontal ligament (PDL) cells and mouse embryonic pre-osteoblasts cells (MC3T3-E1). Both the CBA and coral-HA exhibited a better initial PDL cell adhesion and regarding the long-term PDL cell adhesion there was an increase in the presence of coral-HA. In the tests with MC3T3-E1 cells, the mineral-like nodule formation was significantly higher in DFDD biomaterial. According to the authors, the overall outcome was a good biocompatibility with both types of cell for all the analysed biomaterials [137].

Recognizing that the incorporation additional elements to the scaffolds could improve their properties, Zhang et al. [138] combined silver with coral-HA aiming to introduce antibacterial properties to the scaffold. The scaffolds were prepared through an adsorption process at the surface and ion-exchange reaction between the Ag^{+} from silver nitrate and Ca^{2+} from the coral-HA. It was observed that the scaffolds morphology is dependent on the Ag^{+} concentration. The scaffold cytocompatibility was analyzed by using MC3T3-E1 cells and it was demonstrated that cell morphology and proliferation is dependent on the Ag^{+} concentration, for instance, better resulted were achieved with lower Ag^{+} concentrations [(13.6 μg/ml)/coral-HA and (1.7 μg/ml)/coral-HA]. Importantly, the scaffold that combine silver with coral-HA exhibited an excellent biocidal potential against both Gram-negative (Escherichia coli) and Gram-positive bacteria (Staphylococcus aureus).
As earlier stated, corals are potential scaffolds for bone tissue engineering, however, their excessive use may damage their natural habitats. In this regard, Mahanani et al. [139] mimicked coral structure and studied their capacity for MSCs adhesion and proliferation. The synthetic scaffolds were prepared from bovine gelatin and calcite $CaCO_3$ powder with 10% w/v solid concentration. They observed that MSC exhibited a good adhesion ability and when Platelet Rich Plasma (PRP) are incorporated into the scaffolds, the cell proliferation improved [139].

6.1.1.2. Natural and partial transformed corals combined with mesenchymal stem cells

Manassero et al. [140] studied the potential of Acropora coral scaffolds for MSCs delivery in an animal model. Upon an in vitro cell adhesion and proliferation, the coral scaffolds were placed into a critical bone defect in sheep. They observed an almost complete scaffold resorption 6 months after operation which, consequently, is associated with bone regeneration. The authors conclude that the presence of MSCs is beneficial for the osteoinductive behaviour [140]. A comparation between Acropora or Porites coral granules combined with MSCs for their potential use in bone regeneration was performed by Decambron et al. [141]. The cells were seeded on both types of coral granules, placed in a perfusion bioreactor, and then implanted into bone defects in sheep. They observed that despite an early resorption of coral scaffolds that led to a bone non-union, a superior bone formation

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was registered for Acropora scaffolds. Further, the former scaffold resorb slowly when compared to Porites scaffolds and, thereby, are more closer to the clinical use [141]. Moreover, the osteogenic potential of these two coral species (Acropora and Porites) was compared with β-TCP scaffolds and banked bone in the presence or absence of MSCs. Bone formation was only registered in the samples containing MSCs and the coral scaffolds demonstrated to have the best bone formation capability [142].

The osteogenic potential of human bone marrow-derived MSCs (BMSCs) to induce bone formation even in ectopic sites has been already demonstrated in BMSCs-soaked coral or HA implants in intramuscular pockets in rats [143] and in the repair of critical-sized mandibular defects in large mammal [144,145]. Similar conclusions were drawn by the same research group when coral scaffolds seeded with BMSCs were utilized [146] instead of β-TCP scaffolds used in the previous study [144]. Defects treated with coral alone were used as an experimental control. The engineered bone with coral/BMSCs achieved satisfactory biomechanical properties at 32 weeks postoperation, which was very close to that of the contralateral edentulous mandible. This contrasted with minimal bone formation with almost solely fibrous connection in the group treated with coral alone [146].

Considering that adipogenic and osteogenic cells share part of the early differentiation cascade of MSCs and when compared to BMSCs are easily isolated, in relative abundance and rapidly expanded, adipocytes have been used for the repair and regeneration of different tissues. For instance, Ruth et al. [147] investigated whether adipocytes that have initiated differentiation along one lineage could be converted into osteogenic lineage by merely interacting with marine corals (Porites lutea). Through morphological, histological, enzymatic and quantitative PCR analyses made at different time points (1, 2, 5, 7, 14, 21, and 28 days post-seeding) they demonstrated that preadipocytes could differentiate into bone-forming cells when grown on biomatrix of marine origin without addition of other bone morphogenesis inducers. Following the same line, Cui et al. [148] loaded autologous adipose tissue derived stem cells (ASCs) loaded onto natural coral scaffolds and investigated their potential to a cranial bone defect in a canine model. After 12 weeks of ASCs-coral implantation it was already possible to observe new bone formation and upon 24 weeks 84.19 ± 6.25 % the defect had been repaired, whilst in the control group, coral alone, only 25.04 ± 18.82 % was repaired [148]. In a more recent work, the same authors studied the hypothesis of healing the same defect with allogeneic ASCs seeded onto coral scaffolds without the need of immunosuppressive therapy. They observed that the use of allogenic osteo-differentiated ASCs did not cause any significant systemic immune response or local inflammation and, importantly, micro-computed tomography (micro-CT) analysis demonstrated that the newly formed bone is analogous to that of autologous osteo-differentiated ASCs [149].

The capacity to develop a vascularised coral scaffold seed with marrow-derived osteoblasts was analysed by Chen et al. [150]. Prior to the implantation, the scaffolds were in vitro incubated for two days. To obtain a vascularised biomaterial, the scaffolds were implanted under the rabbit inferior epigastric blood vessels. After two months of operation, it was obtained a well-vascularised scaffold and, in which, by histological analysis it was observed new bone formation [150].

Bensaïd et al. [151] compared natural coral with coral-HA, both were seeded with MSCs. Firstly, in the natural coral scaffolds it was observed that the presence of a pseudo-periosteal layer of MSCs involving the scaffold improved bone formation in comparison with a distribution of MSCs over the implant. Nevertheless, it was observed that due to the high resorption rate, the presence of natural coral scaffolds only led to the bone formation in one sheep. On the other hand, coral-HA combined with the MSCs layer and the autologous bone graft demonstrated to have a similar newly bone formation upon 4 weeks of implantation. Furthermore, after 14 months of implantation, the defects filled with coral-HA combined with the MSCs layer were completely replaced by new bone [151].

Gao et al. [152] wrapped around cylindrical coral scaffolds partially mineralised and strong osteogenic cell sheets that could be obtained from BMSCs under specific cell culture conditions. These cell sheets/scaffolds were implanted into subcutaneous pockets on the backs of mice and bone formation was studied by CT scan and histological observation. Cortical bone was formed within the cell sheet/scaffold. After 8 and 12 weeks of implantations, the bone formation was 26% and 40%,

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respectively. Histological observation showed that neo-bone formation occurs in the manner of endochondral ossification. The authors concluded that a partially mineralised and osteogenic cell sheet could vitalise a coral scaffold for bone formation[152]. Following a similar strategy, Geng et al. [153] combined MSCs sheets and coral particles. Coral particles were incorporated on the surface of confluent rabbit MSCs forming a cell sheet. Posteriorly, a tubular bone graft was obtained by wrapping the cell sheet around a mandrel. The obtained bone graft cultured in a spinner-flask bioreactor and then implanted into subcutaneous pockets on the backs of nude mice. The authors observed that following the in vitro incubation period, the bone graft still exhibit a tubular shape, sufficient radiological density and suitable values of compressive strength. The in vivo results demonstrated that the cell sheets had a good osteogenic capacity and after 8 weeks of implantation a mature tissue with a similar mineral density as the one of mouse spine [153].

In a different approach, Chen et al. [154] inserted a titanium dental implant into a coral scaffold seeded with BMSCs and, then, implanted subcutaneously into nude mice back. Defects treated with coral carrying no cells were used as an experimental control. After 2 months of implantation, the local of implantation was red and similar to native bone and, in addition, the scaffold was mostly absorbed. Also, dental implants were fixed in the newly formed bone and surrounding the implant new bone was formed. On the other hand, no bone formation was observed in the control scaffold [154].

It has been recognized that in supra-alveolar defects, periodontal and bone regeneration can improved when used guided tissue regeneration (GTR) due to the space provision and not to their osteconductive properties [155]. Wikesjö et al. [156–159] different works in which created a supra-alveolar periodontal defect in young adult beagle dogs with the aim to evaluate space provision, alveolar bone, and cementum regeneration using natural corals in combination with GTR. An histometric analysis with different parameters (defect height, defect area, membrane height, junctional epithelium, connective tissue repair, cementum regeneration, bone regeneration (height, area and density) and biomaterial density) demonstrated that among three different step-serial sections there was not significant differences for all the parameters and, thereby a representative histometric data can be obtained from a central section [157]. The combination of coral/GTR was compared with coral [158] or GTR alone [159]. At 4 weeks postoperation the histopathologic and histometric analysis revealed significantly increased bone formation (height and area) in sites receiving the coral/GTR combination compared with coral or GTR alone [158,159].

6.1.1.3. Natural and partial transformed corals combined with mesenchymal stem cells

The capacity of bone tissue to repair and regenerate is in part related to the direct differentiation of MSCs into osteogenic cells. In this process of differentiation, different hormones and differentiation factors play a fundamental role. For instance, transforming differentiation. The osteoclasts have the ability to activate latent TNF-β and, subsequently, during bone resorption the active TNF-β is released and, thereby, promote osteoblast formation and bone formation [160]. Based on this concept, Vuola et al. [161] studied the effect of the TNF-β1 addition to natural coral scaffold in bone formation by adding coral scaffolds with or without TNF-β1 into the defect created in parietal bone of Wistar rats. Indeed, the presence of TNF-β1 improved bone formation, though the new bone was located around the implanted material and did not fill the defect. Furthermore, the addition of the growth factor delayed bone resorption and the authors associated that with the absence of fibrous tissue ingrowth and the decrease of the number of macrophages and giant cells [161].

BMPs are proteins present in bone matrix and are able to induce chondrogenesis and osteogenesis. These molecules are associated with the first signal for the beginning of MSCs differentiation and enhance osteoblast differentiation and osteoblastic differentiation. IGFs are also present in bone matrix and play a role in bone formation. IGFs stimulate chondrogenesis and osteogenesis as well as the synthesis of bone collagen by bone cells [160]. In this regard, Nandi et al. [162] incorporated BMP-2 or IGF-1 into HA scaffolds derived from coralline and used a rabbit model to study their potential for bone regeneration. Through an in vitro study, they observed that IGF-1 exhibited a more sustained release when compared to BMP-2. After 28 days the release for IGF-1 and BMP-2 was 77 and 98%, respectively. The in vivo results demonstrated that the addition of growth

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factors improve the early-stage bone formation, though IGF-1 demonstrated to be more effective [162].

Vascular endothelial growth factor (VEGF) is the best-characterized growth factor for regulation of vascular development and angiogenesis. Since angiogenesis influences osteogenesis, VEGF is important in bone formation and remodelling [163]. Recognizing that the vascularization and osteogenesis of block grafts still remains a key problem for dentists, Du et al. [46-47] attempted to promote angiogenesis and prevascularization by seeding nano-HA (nHA)/coral blocks with angiogenic recombinant human vascular endothelial growth factor$_{165}$ (rhVEGF$_{165}$). Non-coated and coated drafts were implanted in mandibular critical-size defects using male beagle dogs as animal model. The histological evaluation and the histomorphometric analysis revealed enhanced neovascular density and a larger quantity of new bone formation at 3 and 8 weeks postsurgery. The results suggest that nHA/coral blocks might be satisfactory scaffolds for block grafting in critical-size mandibular defects and that additional VEGF coating via physical adsorption can promote angiogenesis in the early stage of bone healing [164,165].

6.1.1.4. Natural and partial transformed corals combined with mesenchymal stem cells and growth factors

A bone tissue engineering strategy that combines a calcium-based scaffold and MSCs is beneficial for bone growth, however may lack osteoinductive properties. This can be overcome by the addition growth factors. For instance, Xiao et al. [166] used autologous BMSCs from Beagle dogs and transfected them with adenovirus containing human BMP-2. Posteriorly, the BMP-2 expressing BMSCs were seeded onto a coral scaffold. The BMP-2-transfected BMSCs/coral scaffolds were placed in defects created in the canine medial orbital wall. The authors observed that a combined delivery of BMSCs and BMP-2 on the defect has the highest bone regeneration when compared with the defects treated with BMSCs/coral and only coral scaffolds [166]. In a similar procedure, Tang et al. [167] transfected autologous BMSCs from the left tibia of osteoporotic rats with human BMP-2 but, instead of seeding them onto a natural coral scaffold, it was used coral-HA scaffolds. The authors reported that 4 weeks after implantation it is observed newly formed bone and mature bone was observed upon 8 weeks of implantation [167]. It is important to highlight that BMP-2 in suprahysiologic doses cause significant adverse side-effects. Decambron et al. [168] combined coral scaffolds with MSCs and/or low-doses of BMP-2 and observed that a dual delivery improved bone formation and bone union when compared to a single addition of MSCs or BMP-2 to coral scaffolds [168]. A comparison between coral scaffolds combined with MSCs and human BMP-2 and autologous bone graft was performed by Hou et al. [169]. At each coral it was firstly added the BMP-2 and, subsequently, MSCs were seeded. The grafts were implanted in critical defects rabbit crania. A similar bone formation was registered in the defects treated with coral/BMP-2/MSCs scaffolds (77.45 ± 0.52% in radiopacity) and with autografts (84.61 ± 0.56% in radiopacity). In addition, the osteogenesis rate in coral/BMP-2/MSCs was significantly higher compared to coral/BMP-2 scaffolds. Thus, it was stated that there is a synergetic effect between MSCs and BMP-2 that were seeded in the coral scaffold and, importantly, these combined scaffolds exhibited a similar behaviour as autologous grafts [169].

bFGF is produced by osteoblasts and stored in ECM in an active form and, thereby it regulates bone remodelling. In addition, bFGF promotes the proliferation of endothelial cells and neovascularization [170,171]. With this in mind, Zheng et al. [172] studied the feasibility of seeding mandibular condyle constructs with BMSCs onto porous coral scaffolds. The human BMSCs were transfected with bFGF gene-encoding plasmids and induced to differentiate into osteoblasts and chondroblasts. The osteogenic/chondrogenic differentiation, cell proliferation, collagen deposition and tissue vascularization were evaluated. They observed that the transfected human BMSCs (h BMSCs) expressed bFGF and were highly proliferative. Moreover, subcutaneous transplantation of seeded coral/hydrogel hyaluran constructs into nude mice resulted in bone formation and collagen type I and type II deposition. Neovascularization was observed around newly formed bone tissue; bFGF expression was detected in implanted constructs seeded with bFGF expressing hBMSCs. Based

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on the encouraging results, the authors suggest that engineered porous coral constructs seeded with bFGF gene-transfected hBMSCs may be a feasible option for surgical transplantation in temporomandibular joint [172].

Zhang et al. [173] prepared porous chitosan/coral composites combined with plasmid encoding PDGF-B gene through a freeze-drying process. PDGF-B is an important growth factor for wound healing as well as promote the recruitment and proliferation of PDL and bone cells [174]. The scaffolds were evaluated in vitro by analysis of microscopic structure and cytocompatibility. The expression of PDGF-B and type-I collagen were detected after seeding human PDL cells in the chitosan/coral scaffold composites. The subcutaneous implantation of these scaffolds into mice revealed that the proliferation properties hPLCs on the gene-activated scaffolds were much better than on the pure coral scaffolds. The expression of PDGF-B and type-I collagen was also superior in gene-activated scaffolds. The results of this study suggest the porous chitosan/coral composite scaffolds combined with PDGF-B gene as potential construct for periodontal tissue regeneration.

Gross-Aviv et al. [175] studied the influence of the coral surface chemistry on the differentiation of MSCs. For this purpose, an aragonite matrix-derived from the coral Poris lutea and a gold coated Poris lutea were seeded with MSCs and combined or not with growth factors (TGF-β1 and IGF-I). An aragonite surface promoted an osteogenic differentiation and, on the other hand, the gold coating that prevented the contact between the cells and the aragonite surface led to a chondrogenic differentiation. The authors stated that the chondrogenic differentiation on the gold-coated scaffolds is associated with the inability of a direct contact between the Ca^{2+} environment of the scaffold and the MSCs. Furthermore, the supplementation of the culture medium with the growth factors increased the influence of the surface chemistry on the cell differentiation [175].

Xiao et al. [176] studied the effects of osteogenic, BMP-2, and angiogenic factors, VEGF, on the repair of critical-sized bone defect in rabbit orbits and, for that, it was used autologous BMSCs genetically modified to express human BMP-2 and VEGF165 and then seeded on natural coral scaffolds. The defects were filled with the coral scaffolds that had been loaded with non-transfected or transfected BMSCs with a single or combined growth factor. After 4 weeks of implantation, in the critical defect filled with the coral scaffolds that had a combined delivery of BMP-2 and VEGF the was an improvement of angiogenesis. Moreover, a maximum rate of bone formation was registered before the 8^{th} week of implantation and a total bone-union was observed at 16^{th} week of implantation. The authors concluded the presence of both BMP-2 and VEGF improved the angiogenesis and bone regeneration. Further, there is a synergetic effect between VEGF and BMP-2 during bone formation [176].

PRP extracted from autologous whole blood contains a wide range of autologous growth factors, namely PDGF, TGF-β, IGF-1, VEGF and bFGF. It is beneficial for tissue healing and is being broadly used in oral and maxillofacial surgery [177,178]. With this in mind, Zhang et al. [179] studied the effect of clotting natural porous coral disks with PRP on the bone formation of marrow stromal cells (MSCs). The samples were cultured in vitro or implanted subcutaneously into nude mice. Coral scaffolds loaded with MSCs or with PRP alone were used as controls. The levels of the ALP, a marker enzyme of bone formation activity was measured for the specimens cultured in vitro for 7 and 14 days, while and the levels of ectopic bone formation were evaluated 4 and 8 weeks after operation. The in vitro results revealed that the samples from the coral/PRP/MSC group exhibited significantly higher ALP activity, compared with those from the coral/MSC group or the coral/PRP group. The histomorphometric analyses of the in vivo experiments also showed higher levels of new bone and/or cartilage formation in the coral/PRP/MSC group, 4 and 8 weeks after implantation in comparison to the control specimens. The authors concluded that PRP could improve the ALP activity of MSCs on coral and increase ectopic bone formation [179].

6.2. Cuttlefish bone

The cuttlefish, Sepia officinalis L., is a common demersal neritic species occurring predominantly near sandy and muddy bottoms up to a depth of 200m. CB represents approximately 9% of the cuttlefish and is a hollow structure divided by lamellae (Figure 8) [22,180,181]. Besides functioning

as skeleton, the porous structure contains liquid and gas (mainly nitrogen at a pressure of about 0.8 atm) that provides neutral buoyancy to the cuttlefish by varying its density [180,181]. Through the liquid movement into or out of the CB via osmotic forces, the volume fraction of gas in the bone changes and the cuttlefish might become more or less dense than the sea water. This buoyancy mechanism, which is almost independent of depth, confers a valuable advantage to the cuttlefish since it can maintain a fixed position in water with little effort [180,182]. This buoyancy mechanism more stable that that conferred by the swim bladder of a fish, which leads to a state of unstable equilibrium when adapting to the depth. Indeed, if the fish rises in the sea, the swim bladder will expand and tend to push it still higher, whereas if it goes deeper in the sea the swim bladder will be compressed and the fish will tend to sink more and more quickly [182]. To accomplish these functional and highly sophisticated requirements, the high porosity of CB enables cuttlefish maintaining its neutral buoyancy at a higher depth and at the same time retain enough stiffness and strength to prevent sever distortion or crushing under high hydrostatic pressures in deep water [180,183,184].

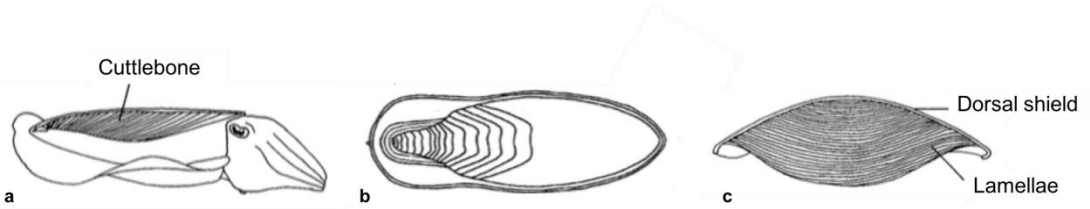

Figure 8. Schematic representation of: **(a)** the CB disposition within the cuttlefish; **(b)** the CB; **(c)** a transverse section through the CB. Adapted from [99,180].

Dorsal shield and lamellar matrix represent the two main components of CB (Figure 9). The dorsal shield is highly dense and provides a rigid substrate for protecting the development of the lamellar matrix. On the other hand, the lamellar matrix has a high porosity, approximately 93%, and also manages to withstand very high hydrostatic pressure [184].

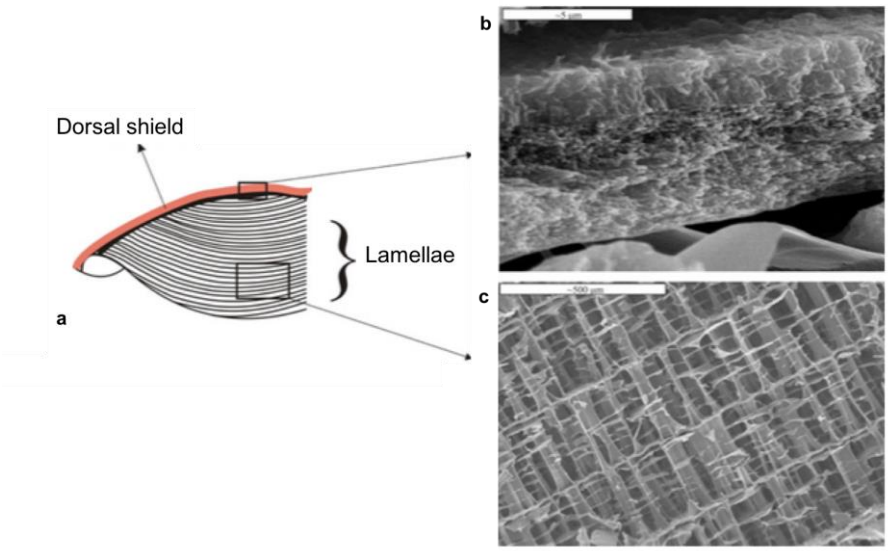

Figure 9. CB microstructure: **(a)** Schematic representation of a transverse section of CB; **(b)** Scanning electron microscopy (SEM) of dorsal shield; **(c)** SEM of lamellar matrix. Adapted from [185].

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The dorsal shield is a dense and tough cover that overlays the lamellar matrix and seals off the separated chambers. It consists in a non-porous structure containing 30 to 40% by weight of organic material, β-chitin. This organic component has the ability to undergo plastic deformation, thereby increasing the toughness of the structure. It is composed of three different layers: a 1 mm thick outer calcified layer, a 0.3 mm thick transparent middle zone composed of tough, fibrous layers of sclerotized chitin, and a thin inner calcified zone [180].

The lamellar matrix is a layered, quasi-periodic microstructure constituted by lamellae separated by pillars [184] , which is able to resist external pressures of about 2.4 MPa [180]. The spacing of the lamellae varies between 200 and 600 μm along the CB [180], being dependent on the specie [186] Each chamber is supported by pillars that form channels with a high degree of interconnectivity, and a uniform width that progresses through the material along a sigmoidal path [184]. Its unique macroporous architecture with approximately 93% of porosity is mainly composed of aragonite, which is a crystallized form of $CaCO_3$. This inorganic structure is enveloped in a thin layer of organic material composed primary of β-chitin that represents between 3 and 4.5% of the lamellar matrix weight [180,184,187]. The presence of β-chitin is associated with initiation, organization and inhibition of the inorganic matrix mineralization [180,187]. Moreover, organic matrix may function as a template for the nucleation of the inorganic material possibly due to the interaction between the groups of the polymer and the ions in the solution phase or the nuclei of the solid phase that become more stable upon bonding [180].

CB has a unique architecture which is associated with a high ratio of compressive stiffness to weight. Indeed, under an excessive compressive loading, CB can collapse in a controlled and layer-by-layer manner due to its layered structure and also to the sigmoidal shape of its pillars. In this regard, cuttlefish can stay below the depth limit of its CB for short periods of time without a significant damage. The plastic deformation of the organic component in the dorsal shield provides another important contribute to the toughness of the structure [180,184].

6.2.1. CB-derived bone grafts substitutes

CB is a worldwide available and inexpensive material with a unique porous microstructure, the pore size and pore interconnectivity of which is proven to be beneficial for bone growth and vascularization [188]. The use of CB as bone graft substitute has been reported not only in its natural form but also as an aragonite source to prepare CaP materials by preserving the same porous structure after hydrothermal transformation.

6.2.1.1. Natural CB

Dogan et al. [189] studied the potential of CB as a xenograft by filling a bone defect of a rabbit with CB, demineralized bone matrix (DBM), bovine cancellous graft (BCG), and TCP. At the third postoperative week, it was observed 100% callus formation in the groups treated with CB, DBM and TCP. In the animal treated with CB, remodelling could be observed at week 6, while in the in the other groups remodelling was only observed upon the twelfth week. In addition, in the group treated with CB, fibrous union started at the first postoperative week and the rate of vascularization was higher when compared to the other groups. The osteochondral union at week three resulted in increased of the development of new bone. The defects treated with CB or with TCP exhibited the fastest remodelling. Overall, in the CB group it was observed a high degree of vascularization and good osteogenesis and osteointegration properties. Thus, CB was considered a good xenograft material [189].

As previously mentioned CB is composed by a dorsal shield and lamellar matrix. Kim et al. [190] separated the CB into these two structures and studied if the presence of small amounts of heavy metals on CB could influence its biocompatibility and osteoblast differentiation of human MSCs. It was observed that the heavy metals did not affect the cell viability and CB allowed the cell adhesion and growth. When compared to the dorsal shield, in the lamellar matrix the cells infiltrated deeper into the structure. ALP levels, increased on both dorsal shield and lamellar matrix, however it has a higher expression on dorsal shield due to the fact that confluence is achieved earlier in this material

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and this, consequently, resulted in earlier differentiation. Collagen type I α1 (ColIα1) and Runx2 was increased on both materials with no significant difference [190].

Besides being used as a scaffold, natural CB can also be milled and used to fill poly (methyl methacrylate-co-styrene) bone cements [191,192]. When compared with the non-filled cement, the addition of 10 and 30 wt% of CB resulted in an increase of tensile strength and Young's modulus. Nonetheless, a cement containing 50 wt% of CB led to a decrease in tensile strength and Young's modulus, which was associated with the formation of pores. The compressive strength although has decreased with the increase of CB content, was maintained higher than the minimum required of 70 MPa [191]. Due to the exothermic polymerization reaction, there is an increase on temperature as reaction progresses, decreasing after a maxim value has been reached as the monomer is consumed. Due to its thermal capacity, the increase of CB content led to a decrease of the peak temperature and an increase of the time to reach that temperature, resulting in an apparent slower reaction. Furthermore, the setting time increases with the CB content, which is beneficial to allow enough manipulation time before setting. However, a longer setting time can cause some medical problems because pressure in the prosthesis has to be maintained until the cement sets [192]. When implanted in bone defects, the cements without CB were weakly adherent to the parietal bone while the samples containing CB were strongly attached to the bone, an indication that osteointegration has occurred in samples containing CB [191].

6.2.1.1. Calcium phosphate materials derived from CB

Being mainly composed of aragonite and having a suitable porous microstructure for bone grafting, which is preserved upon hydrothermal transformation into CaP, CB has been used as an interesting source of calcium carbonate and template for preparing CaP scaffolds or powders.

The transformation of CB into HA through a hydrothermal process was firstly reported by *Rocha et al.* [122,188,193]. Prior to the transformation, the exact aragonite content in CB was analysed and the corresponding amount of $(NH_4)_2H_2PO_4$ was added to set the molar ratio of Ca/P = 1.67. The mixture was sealed in polytetrafluoroethylene (Teflon) lined stainless steel autoclave and the hydrothermal treatment (HT) was conducted at 200°C. The CB internal structure was not compromised during the HT and thereby the pore size and interconnectivity was maintained after the transformation [122,188,193]. It was obtained a AB-type carbonated HA, which is similar to the composition of human bones [122,193]. Additionally, the size of HA crystallites was similar to the size of bone-like apatite (20-50 nm) [122]. The obtained scaffolds demonstrated to have a high thermal stability on sintering up to 1350°C. Above 1400°C was possible to observe the formation of β-TCP. Moreover, scaffolds demonstrated to have a very good *in vitro* bioactivity due to the fact that when immersed in simulated body fluid (SBF) there was a rapid and evident formation of a HA layer [188]. In the presence of the scaffolds the viability and proliferation increased, and the ALP activity was maintained constant indicating that the scaffolds were biocompatible with osteoblasts [188][193].

Different HT conditions were established by Hongmin et al. [194], who used a 0.5 M $(NH_4)_2HPO_4$ solution and a CB/$(NH_4)_2HPO_4$ weight ratio of 1/1.2 and the treatment was conducted at 180°C for 96 h. The authors compared the *in vitro* and *in vivo* behaviour of HA scaffolds derived from CB (CBHA) and raw CB. The protein adsorption was higher on CBHA than on CB. Despite the good cell adhesion and proliferation on both CBHA and CB, CBHA demonstrated to have a better capacity for cell differentiation. Indeed, after 13 days of culture, ALP activity and osteocalcin (OC) level was significantly higher on CBHA. Regarding the *in vivo* behaviour, both scaffolds were encapsulated in a fibrous tissue at the fourth week, however higher quantity of fibrous tissue and blood vessels were observed on CBHA. At week eight and contrarily osteoblasts were found to excrete bone matrix and embed themselves to form bone lacunae. In this way, it was possible to conclude that CBHA represents an interesting osteoinductive bone graft [194].

As previously observed different HT approaches can be followed to obtain CaP materials from CB. In this regard, *Ivankovic et al.* [185] and *Kasioptas et al.* [195]. studied the mechanism and kinetics of the aragonite transformation into CaP scaffolds by modifying the temperature and time of the hydrothermal reaction. *Ivankovic et al.* [185] analysed a range of transformation temperatures from 140 to 220°C for various time periods between 1 and 48 h. In the samples treated at 140 and 160°C for 20 min they observed the presence of $CaHPO_4.2H_2O$ (brushite), a compound that preferentially precipitates at lower values of pH and temperature. A complete transformation was only observed upon the HT at 200°C for 24 h. At 180°C the transformation is not completed

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even after 48 h of HT. On the other hand, the formation of $CaHPO_4$ (monetite) was reported to occur at 220°C and above 4 h of HT. The isothermal kinetics of transformation was described by the Johnson-Mehl-Avrami (JMA) equation (Eq. 1), which is often used to describe the nucleation and growth of crystals from amorphous materials.

$$\alpha = 1 - exp[-(k^n\ (t - \tau)^n)] \quad (1)$$

In Eq. 1, α represents the fraction of transformed HA, k is the rate constant, n is the Avrami exponent, associated with the nucleation type and growth dimensions, and τ is the incubation time. The rate constant increased with the increase of temperature, however, the Avrami exponent was maintained almost constant and around 0.5 over the entire range of temperatures tested indicating that the growth mechanism of HA can be defined as one dimensional growth controlled by a diffusion process [185].

Alternative methods to HT can be used to produce CaP materials derived from CB. For instance, *Sarin et al.*c[196] reported the production of BCP scaffolds from CB using a solution of phosphoric acid and 2-propanol with different concentrations of phosphoric acid from 12 to 20wt%. Briefly, the infiltration of the solution into the CB occurred under vacuum conditions and for 1 h. This process was followed by heat treatment at high temperatures, up to 1300°C. The BCP scaffolds obtained with 16 wt% of phosphoric acid preserved the initial microstructure of CB and exhibited a compressive strength of 2.38 ± 0.24 MPa that despite being lower than the raw CB (2.74 ± 0.39 MPa), is in the range of trabecular bone [196]. *Dutta et al.* [197] produced the CaP scaffolds derived from CB in ambient conditions using a de-calcification, re-calcification process followed by a phosphate mineralization. $CaCO_3$ was removed from the CB structure by dipping the CB in acetic acid. The as-obtained organic matrix was re-mineralized using a solutions of calcium chloride and sodium carbonate and hence amorphous calcium carbonate (ACC) was produced, due to the fact that macromolecules associated to biominerals have the capacity to stabilize ACC. Afterwards, ACC was converted into calcium phosphate by immersing the mineralized scaffold into a phosphate solution followed by a treatment with glutaraldehyde solution. Since the solubility of ACC and calcite at the working pH of 7 is high, there was a complete phase conversion, not to HA but instead to brushite, the formation of which is favoured at this pH [197].

In addition to being converted in porous CaP scaffolds preserving the internal structure, CB can also be used to produce CaP powder [198,199] or granules [200]. Lee et al. [198] mixed CB that was previously calcined, consisting of pure CaO phase, with phosphoric acid to synthesize CaP. Different ratios between CB and phosphoric acid from 1:1.0 to 1:1.7 were tested. HA and β-TCP were obtained at 1:1.2 and 1:1.7 ratios, respectively. The samples heat treated at 900°C were entirely crystalized [198]. CBHA granules with a size varying from 200-500 μm were obtained by HT at 200°C for 24 h. Besides of being non-toxic, the CBHA granules enabled a higher cell density and improved cell differentiation capacity in comparison to pure HA granules. This could be justified not only by the higher surface roughness and surface area of CBHA that are beneficial for cell proliferation and adhesion, but also because CBHA contains some mineral ions, like magnesium, that play fundamental roles in the binding of cell-surface receptors and ligand proteins that, consequently, can enhance cell attachment. The *in vivo* results were in agreement with the *in vitro* tests and CBHA had a significantly higher percentage of bone formation and more multi-nucleated giant cells, fibroblasts, osteoblast-like cells and connective tissue and micro blood vessels[200]. Furthermore, *Kim et al.* [199] produced a porous composite scaffold by solvent casting and particulate leaching using CBHA powder and PCL. CBHA powder, obtained by HT at 200°C for 24 h, and PCL were mixed with salt particles (200-300 μm) that acted as a porogen. The introduction of CBHA into the PCL scaffolds improved the compressive strength. Regarding the *in vitro* tests, the addition of CBHA improved the viability, adhesion and proliferation. In the *in vivo* studies it was demonstrated that CBHA/PCL scaffolds improved the bone formation [199].

6.2.1.2. Doped - calcium phosphate materials derived from CB

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The inorganic part of the human bone consists of carbonated HA with trace amounts of other ions like Na^+, Mg^{2+}, Zn^{2+}, Sr^{2+}, F^- and silicon. Therefore, the incorporation of the aforementioned ions into the calcium phosphate matrix has been used to improve the biological performance of the material. With this in mind, Kannan et al. [201] and Kim et al. [202] incorporated F^- and silicon (Si) ions into the CBHA structure, respectively.

F^- is an important element for bone and dental growth. It is involved in the prevention and treatment of dental caries. The incorporation of F^- ions into the HA structure stimulates the osteoblast proliferation and differentiation and improves the mineral deposition in cancellous bone [203–205]. Two different levels of F^- substitutions (46% and 85%) on the OH^- sites via HT at 200°C for 24 h were studied by Kannan et al. [201]. For the HT a required volume of $(NH_4)_2HPO_4$ was added to the CB samples setting a Ca/P molar ratio of 10/6 and F^- was added by the NH_4F solution at 1 M or 2 M. A nano-sized AB-type carbonated apatite with a precise control of F^- content was obtained. For a substitution of 85%, the lattice parameters were the typical for fluorapatite, leading to a contraction along the a-axis.

Silicon represents an important trace element in bone, as it is associated with bone growth. The incorporation of Si into HA materials modifies its surface by creating a more electronegative surface, generating a finer structure and also increasing the solubility. Furthermore, Si-substituted materials demonstrated to improve the bioactive behaviour and increase the bone formation in vivo [92,205]. Kim et al. [202] synthesized Si-substituted CBHA using hydrothermal and solvothermal methods. Firstly, CB and $(NH_4)_2HPO_4$ were placed in an autoclave for HT at 200°C for 6 h. Subsequently, CB was immersed in a solution of silicon acetate ($Si(CH_3COO)_4$) saturated with acetone and solvothermal treatment took place at 200°C for 12 h. In the final step, CB was again mixed with a $(NH_4)_2HPO_4$ solution and the HT occurred at 200°C for 12 h. The Si content on the samples was about 0.77 wt%. Regarding the in vitro results, the presence of a Si-OH layer on the biomaterial surface was associated with the improvement of cell adhesion and proliferation on the Si-substituted CBHA. Moreover, the incorporation of Si enhanced cell density, and consequently improved cell differentiation, which was observed by the ALP activity and expression of Runx2, ColIα1 and OC. According to the in vitro results, the in vivo tests demonstrated that bone formation was higher on Si-substituted CBHA than on CBHA [202].

6.2.1.3. Composite materials derived from CB

CaP scaffolds obtained from CB are normally brittle and exhibited low strength, thereby limiting the application of these materials as bone grafts. In order to improve the compressive strength, some authors investigated the coating of these scaffolds with polymers like PCL [206–209], polyvinyl alcohol [207] and collagen [210].

CBHA scaffolds were obtained through a HT and PCL was incorporated into the scaffold structure under vacuum to remove the air from the pores and allow a complete impregnation of the polymer into the structure [206–209]. Kim et al. [206] reported the coating with 1, 5, and 10% of PCL of CBHA scaffolds. The PCL layer obtained by the coating with 1% PCL could be hardly observed and the roughness of the sample was similar to the uncoated one. Coating with 5% PCL led to the formation of a thin PCL layer that covered the entire surface. With further increasing the amount of PCL to 10%, a thicker layer was formed and the number of clogged pores increased, thereby decreasing the porosity [206]. Nonetheless, Milovac et al. [208] coated the scaffolds with 20% PCL, and although roughness and porosity have noticeably decreased, they reported that such a coating allowed the maintenance of the number of pores and their interconnectivity [208]. Through SBF assay, it was possible to observe the formation of an apatite layer on the PCL coated scaffold [207]. The compressive strength decreased with the HT due to a more extensive degradation of the organic matter that provides the mechanical support of the structure, however, with exception of the coating with 1% PCL, the compressive strength improved with the PCL coating as reported in Table 2. The coated scaffolds did not exhibit any cytotoxicity, cells were able to adhere and proliferate and also it was demonstrated that penetration of cell happened through the entire depth of the scaffold [206,209]. The cell proliferation was higher for the sample coated with 1% PCL. Furthermore, for the

sample coated with 10% PCL the cell proliferation decreased after 6 days of culture due to the reduction of porosity and the consequent less surface available for cell adhesion [206]. The ALP activity increased on the scaffolds coated with 5%, 10% and 20% PCL [206,209]. The expression of ALP, Runx2 and ColIα1 significantly improved with the coating of 10% PCL, since the cells reached the confluence earlier, which consequently beneficiated the differentiation of cells [206]. The scaffolds coated with 20% PCl exhibited a higher level of collagen production, suggesting that PCL coating has improved cell adhesion and consequently induced higher levels of collagen secretion [208].

Table 1. Main calcium orthophosphate componds [85,89–91].

Ref	Compressive strength (MPa)					
	Raw CB	CBHA	CBHA-1%PCL	CBHA-5%PCL	CBHA-10%PCL	CBHA-20%PCL
[206]	1.63 ± 0.13	1.11 ± 0.26	1.25 ± 0.56	2.32 ± 0.44	3.67 ± 0.46	—
[207]	0.609	0.376	—	1.376	—	—
[208]	0.46 ± 0.06	0.15 ± 0.09	—	—	—	0.88 ± 0.11

Siddiqi et al. [207] reported the coating with 5% of PVA of CBHA. The addition of 5% PVA improved the mechanical strength to 0.95 MPa compared to the raw CB (0.61 MPa) and the hydrothermally transformed CBHA scaffold (0.38 MPa). The coating with PVA did not modify the viability and the adhesion and proliferation capacity of CBHA scaffolds [207].

Alternatively to the production of CBHA through HT and a posteriorly coating with polymer, Sukul et al. [210] modified the raw CB with HA and collagen (CB-HA-COL). To this end, CB was immersed in SBF solution to form an HA layer and subsequently samples were coated with collagen through the freeze-drying method. The modification of the CB surface with HA and collagen resulted in a lower values of pore size (80-100 μm) and porosity (~84 %) but still these values have been reported as beneficial in bone tissue engineering. On the other hand, the coating enhanced the compressive strength from 2.00 ± 0.40 MPa of raw CB to 2.71 ± 0.16 MPa of CB-HA-COL. The coated scaffolds promoted cell adhesion and proliferation and showed higher levels of ALP expression [210].

7. Conclusions

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Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "X.X. and Y.Y. conceived and designed the experiments; X.X. performed the experiments; X.X. and Y.Y. analyzed the data; W.W. contributed reagents/materials/analysis tools; Y.Y. wrote the paper." Authorship must be limited to those who have contributed substantially to the work reported.

Conflicts of Interest: Declare conflicts of interest or state "The authors declare no conflict of interest." Authors must identify

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