Hydroxyapatite Particles—Directing the Cellular Activity in Bone Regeneration Processes: An Up-To-Date Review

Denisa Alexandra Florea 1, Cristina Chircov 1* and Alexandru Mihai Grumezescu 1,2,*

1 Faculty of Applied Chemistry and Materials Science, University Politehnica of Bucharest, 011061 Bucharest, Romania; denisa.florea94@yahoo.com (D.A.F.); cristina.chircov@yahoo.com (C.C.)
2 Research Institute of the University of Bucharest (ICUB), University of Bucharest, 060101 Bucharest, Romania
* Correspondence: agrumezescu@upb.ro; Tel.: +40-21-402-39-97

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Abstract: Tissue engineering has evolved quickly over the years and provided three generations of scaffolds for bone tissue applications. Numerous materials have been used to induce the desired effect at cellular levels. Mechano-transduction is a phenomenon which is now gaining the attention and focus of tissue engineering researchers. The idea of controlling the cellular fate and inducing a proper response of the human body in contact with different tissue-engineered systems is now under investigation. Moreover, in order to avoid the appearance of on-site infections and the need for a second surgery, scaffolds with dual functionality are now being developed. Hydroxyapatite (HA) is an intensively studied material in this field and various combinations are under examination for the development of such scaffolds. Various techniques were exploited over the years for HA scaffold production, in order to obtain the most accurate matrix which can mimic the native bone tissue and restore its function. Biomimetic scaffolds aim to direct the cellular fate by imitating the natural structure of the bone tissue in terms of porosity, topography, composition, and surface properties. HA particles are exploited in bone tissue engineering in many forms, such as pure or composite scaffolds or reinforcement agents. In this regard, the aim of this review is to offer a current state of art about the use and synthesis of hydroxyapatite particles and their interaction with the physiological media under certain circumstances.

Keywords: tissue engineering; scaffolds; bone tissue; hydroxyapatite; mechano-transduction; cell signaling; inorganic particles

1. Introduction

When compared to other human body tissues, bone tissue is a distinctive tissue in terms of regeneration and self-repair. However, the process of bone healing might be restricted by a series of factors which may include on-site infections, location of the defect, depth, width, or other individualities of trauma, disease, immune system performance, or patient particularities. According to recent estimations, the incidence of bone fractures is constantly growing, while the rate of healing failure is more than 10%, especially in patients that suffer from age-related disorders, genetic diseases, malformations, or even bone tissue diseases (e.g., osteoporosis, rheumatoid arthritis) because all of these factors are restricting the self-healing ability of the tissue [1,2].

For instance, the International Osteoporosis foundation stated that every 3 s a fracture caused by osteoporosis occurs worldwide, with a total of around 9 million annually. The total number of patients affected by this disease was estimated to be around 75 million in Europe, United States of America, and Japan. Moreover, there is a high disability associated with osteoporosis, having a
major impact on the economic sector of each country as less patients are able to work or continue with their daily activities [3,4]. The treatments available on the market are based on medication which aims to decrease the risk of fracture, including bisphosphonates (risedronate, zoledronic acid), teriparatide, romozumab, denosumab, or raloxifene. However, the risk of fracture and massive bone loss is still high, the osseointegration of an implant or prosthesis is difficult, and the native regeneration processes are slow and difficult [5]. These issues are also applicable in other bone diseases, for example, in patients with arthritis, where the incidence is one in five. Arthritis affects the joints by damaging the cartilage causing bones to start to press on each other, leading to bone loss, fractures, and disability [6]. Small bone injuries are usually stable and benefit from spontaneous native healing without the need for surgery. However, injuries correlated with bone loss lead to inadequate alignment of bone structures and present high instability, therefore the need for surgical intervention becomes a must. Bone fracture healing occurs by numerous events starting with hematoma formation, followed by inflammation and cartilaginous structure creation, angiogenesis, and mineralization. This process is complicated in large bone defects and, when other pathologies are present, complete fracture healing is almost impossible to achieve [7].

In such cases, bone grafts are used to fill the defects caused and speed up the healing process. The use of a proper bone graft is correlated with multiple aspects, including defect nature and size, graft characteristics (size or shape), biomechanical performance, physiological conditions, handling, manufacturing process, production costs, or ethical principles [8]. Three main classes of bone grafts can be distinguished: allografts, xenografts, and autografts [9]. While autografts present important osteogenic characteristics with a key role in bone healing and remodeling processes and represent the ‘gold standard’, their use has been associated with a variety of drawbacks, such as donor site morbidity, injuries at the harvesting site, or severe pain. On the other hand, xenografts and allografts present good osteoinduction and osteoconduction, but both of them present a deficient osteogenic performance. Moreover, xenografts and allografts showed high rejection rates and increased risk in disease transmission [10]. Therefore, tissue engineering strategies based on the use of biomaterials as scaffolds for filling bone defects have attracted a great interest. Additionally, the socio-economic outcomes together with the long-term results obtained by using currently available treatments for bone-associated conditions have motivated researchers to consider nanotechnology and bone tissue engineering promising ways for developing more efficient and cost-effective solutions [11–14].

The aim of this review is to offer a view of current state of the art about the use and synthesis of hydroxyapatite particles and their interaction with the physiological media under certain circumstances.

2. The Importance of Bone Remodeling, Osteoinduction, and Mechano-Transduction Phenomenon in Scaffold Design

In general, tissue engineering is an approach which involves multiple science domains, such as biology, engineering, medicine, chemistry, and physics, so as to create complex structures which encourage and intensify the human body’s response to an injury [15]. In bone tissue engineering, the main scope is to restore the native function of the bone tissue in the case of fractures or diseases. One of the most essential components of such structures is represented by scaffolds which are matrices that act as a platform for guiding the cellular response and, consequently, tissue formation. There are numerous considerations which need to be taken into account when developing a scaffold for bone tissue applications including, but not limited to, native bone structure, biomechanics, pore characteristics, mechanical strength, chemical properties, and manufacturing technique [16]. Over the last decades, a variety of techniques have been used with the purpose of developing highly complex bone scaffolds, e.g., solvent casting, 3D printing (additive manufacturing), chemical foaming, and foam-gel [17–19].

As previously stated, one current challenge of orthopedic implants, protheses, or other treatments is represented by the high rate of local infections which may lead to serious pain and complications and consequently to a second surgical intervention, amputation, or, in the worst case, the death of the patient. This is a major drawback considering both patient recovery and the economic impact on
the healthcare system. Therefore, the development of a strategy which presents dual-functionality is needed. Bone tissue engineering aims to acquire a system which can direct the cellular response in the human body and present antimicrobial properties. Such a system will potentially induce osteoinduction and limit the use of antibiotics after surgery. Thus, the materials chosen for bone scaffolds have a considerable impact on the final system performance [20,21].

The main mechanism that offers the ability of self-regeneration in bones is the bone remodeling process, which is a permanent process based on bone resorption (regulated by osteoclasts) and bone formation (regulated by osteoblasts). However, there are many cells involved in this process (e.g., osteocytes, lining cells) all performing together in a manner which assures a stable equilibrium between resorption and formation [12]. Bone remodeling occurs in five distinct stages (Figure 1), starting with the resting state in which the tissue surface is covered by cells that are not active and followed by the activation stage where two types of stimuli (physical or hormonal) guide the migration pathway of macrophages and monocytes to the remodeling site where they are subjected to osteoclastic differentiation. In the meantime, the third stage is activated and the osteoclasts start to remove both inorganic and organic components of the bone and form a cutting cone in the compact bone and a so called “Howship’s lacuna” (a cavity in the trabecular bone). This is important because when the cavity reaches a certain dimension and shape in each type of bone the resorption stage stops. The fourth stage is called reversal and it starts when osteoclasts disappear and the cells which migrated in the second stage deposit a substance on the bone surface which is very close in structure to a cement. The aim is to smooth the surface and facilitate interactions between the old and new bone. Additionally, the activity of pre-osteoblasts starts in this stage as well. Finally, the fifth stage is the bone formation process in which osteoblasts start to fill in the above-mentioned cavities and to produce the osteon in two main phases [22]. In the first phase, collagen-I and other constituents of the osteoid are deposited, which are then mineralized in the second phase. This complex process is very important as it also sustains the mineral homeostasis and strength of the bones, allowing them to uphold massive loads. Moreover, it assures regeneration in the case of micro-cracks through the intervention of osteoclasts which resorb the damaged sites and osteoblasts which form the new bone tissue [12,22]. The regulation of the bone remodeling process is strongly influenced by the mechanical environment, which controls osteogenesis due to bone cells response to direct or indirect mechanical stimuli [23,24].

![Figure 1. The five stages of bone remodeling.](image-url)

When it comes to bone healing, there are three main processes which need to be taken into account when designing a scaffold: osteogenesis, osteoconduction, and osteoinduction. While osteogenesis is the physiological process through which new bone is synthetized by specific cellular constructs, osteoconduction and osteoinduction are responsible for the migration and differentiation pathways of the cells. In this manner, osteoinduction recruits stem cells at the desired site and dictates their osteoblastic differentiation, while osteoconduction involves the ingrowth and spread of the
bone-forming cells onto a surface. These two processes are highly exploited in terms of scaffold design due to the desire to create a system that can induce a native response and to adapt to the surrounding tissues for a better and faster healing [25–29].

Considering all these aspects, it can be stated that in the case of fractures, defects, or trauma, scaffolds need to be able to induce the attachment and migration of the cells. In this regard, it is very important to understand the basic mechanisms of the extracellular matrix (ECM), which is a non-cellular constituent in all human body tissues and organs that links cells together as tissues, and acts like a “filler” between them [23]. The ECM is composed of two main categories of macromolecules (fibrous proteins and proteoglycans) (Table 1) that are gathered together as a complex and organized setup. In essence, the ECM is a dynamic structure that confers many characteristics to a tissue, such as compressive and tensile strength and elasticity, and it can even protect tissues through homeostasis maintenance. Over time, research has shown that there is a variety of organic and inorganic compounds that impact bone development processes and are categorized into matrix-attached molecules (e.g., osteocalcin, fibronectin, alkaline phosphatase, osteonectin, osteopontin, thrombospondin) and soluble factors (e.g., fibroblast growth factor, platelet-derived growth factor, calcitonin, thyroxin, estrogen) [22,23].

Furthermore, cellular adherence to the ECM is intermediated by specific receptors, including syndecans or integrins, which are also known as “mechano-sensors” [30]. The integrin family is a class of receptors that is composed of heterodimers of alpha and beta subunits, both of them comprising type-I transmembrane proteins with short cytoplasmic tails [27]. Generally, these receptors are involved in two main processes, namely cell–matrix and cell–cell interactions. In a normal physiological environment, mechanical inputs received by internal pathways (for instance, from muscle forces) and the ones received from external sources (such as gravitational force) are sensed and converted by integrins into molecular signals which lead to the development of specific architectures and, consequently, to bone formation and tissue organization [30–32]. This process is known as mechano-transduction and, most probably, the majority of eukaryotic cells are mechanosensitive, playing a key role in the growth and regeneration of all the living tissues. Essentially, there are three main components involved in this mechanism: focal adhesion, mechanosensors, and signaling specimens which are able to generate changes at gene levels and protein expression profiles. However, this is a mid-term process, with a timescale that varies from seconds to several weeks. The first stage is the activation of mechanosensors which occurs in a few seconds, followed by the generation of genetic changes in the following hours, the directing of cell functions in the following days and finally, permanent changes in cellular phenotype and differentiation pathways in the following weeks [30]. It was demonstrated that cells are able to react to a multitude of mechanical cues, including static ones, such as mechanical or chemical properties (e.g., surface biochemistry, topography, or stiffness). One key element in mechano-transduction is the integrin-mediated signaling as a result of being the core receptors for ECM-related proteins such as fibronectin, laminin, or collagen. As previously stated, integrins are made of subunits, 18 alpha and 8 beta subunits that have their own affinity for multiple peptides. The intracellular part of integrins is in continuous interaction with cytoplasmatic proteins making possible the development of focal adhesion complexes that are able to provide a direct connection between the outer matrix and the cytoskeleton [33–35].

Table 1. The main components of bone organic extracellular matrix (ECM) [36,37].

| Collagens            | Non-Collagenous Proteins          | Proteoglycans/Glycosaminoglycans |
|----------------------|-----------------------------------|----------------------------------|
| Collagen type I      | Osteonectin (SPARC)               | Decorin                          |
| Collagen type III    | Osteocalcin (bone-Gla protein)    | Lumican                          |
|                      | Osteopontin                       | Biglycan                         |
|                      | Sialoprotein                      | Epiphycan                        |
|                      |                                   | Keratocan                        |
Taking all these aspects into account, research has been focused in the last decades on controlling the stem cell niche in tissue engineering, and on promoting the desired cellular response for various cellular phenotypes. This strategy seemed to be promising in terms of guiding the tissue response in contact with specific stimuli in various fields. Therefore, the scaffold design should be focused on multiple aspects including, but not limited to, material type, surface, topography, architecture, antimicrobial properties, chemical and mechanical properties, and ability to induce the bone forming processes. Commonly, the manufacture of a tissue-engineered bone consists of three components: scaffold, stem cells, and molecules which can direct the cellular activity (i.e., growth factors) [38,39]. When it comes to scaffold construction, a variety of materials have been used over time for bone repair purposes, such as ceramics, polymers, bioglasses, and hybrid or composite materials. However, a biomimetic scaffold should consist of an inorganic component which is able to mimic the ECM architecture, should present high porosity for promoting cellular anchorage and proper degradation rate, and should encourage bone formation and osteoblastic differentiation (to be osteoinductive) [40].

3. Hydroxyapatite Particles in Scaffold Manufacturing and Cell Signaling

Intensive research has been carried on finding a suitable alternative for the proper healing of bone tissue. Metallic implants started as bioinert devices accepted by the host and after decades scientists were focused on how they can actively influence the actions of the surrounding tissues. The same pathway was also followed in the case of scaffolds, the first generation being composed of bioinert scaffolds, the second one was represented by bioresorbable and bioactive scaffolds, and presently, the third generation under investigation is the one of bioinstructive scaffolds which are meant to stimulate a particular cellular response [38].

One of the most exploited classes of materials in bone tissue applications is represented by ceramics which already have a broad range of applications available on the market. In terms of properties, ceramics stand up owing to their high hardness, low conduction of electricity, porosity, and their enhanced corrosion and compression resistance. Additionally, their surface characteristics are able to promote the adhesion of a wide range of molecules and cells [41].

First of all, one of the most discussed ceramics in hard tissue area is hydroxyapatite (HA), the mineral constituent of the tissue, which is an inorganic compound able to trigger both osteoconduction and osteoinduction. The structure of natural HA presents multiple trace elements, such as Mg²⁺, Si²⁺, Ba²⁺, K⁺, Na⁺, F⁻, Zn²⁺, and CO₃²⁻. When compared, it can be stated that non-stoichiometric (natural) HA is deficient in calcium (Ca) or phosphorus (P), while stoichiometric (synthetic) HA has a molar ratio of 1.67, exhibiting a beneficial effect on bone regeneration processes [42]. Synthetic HA (Ca₁₀(PO₄)₆(OH)₂) proved to be highly biocompatible with a chemical composition comparable to the native tissue. Furthermore, its bioactive and osteoinductive characteristics make it highly suitable for a multitude of applications. A variety of methods have been developed over time to obtain HA-based scaffolds, including solvent casting, gas foaming, electrospinning, 3D printing, or freeze-drying techniques. The bioactivity of HA has been demonstrated over the past decades [43–45]. Additionally, it has been observed that porosity gives an osteoinductive behavior to the scaffold, mediating the interactions at ECM levels. Therefore, investigation has been focused on developing porous scaffolds, with the help of different substances which can change the shape, dimension, or architecture of the pore matrix [13].

3.1. Porous HA-Based Scaffolds

In 2018, Ren et al. created HA scaffolds with micropores that had microchannels on the surface. The aim of these so-called HA scaffolds with grooved structure was to increase the adhesion of proteins at the surface and to induce bone formation and vascularization. The scaffolds were placed in contact with human placenta-derived mesenchymal stem cells, exhibiting high osteoinductive performance by promoting osteoblastic differentiation and proliferation. Moreover, tests conducted on in vivo models established that four weeks after implantation, a thin calcified bone layer was formed, and the
appearance of connective tissues and blood vessels was observed. Nevertheless, various methods have been explored in order to obtain a proper porosity and a suitable surface chemistry, such as the addition of inorganic, organic, or porogen agents into the scaffold structure [43].

Furthermore, Chen et al. stated that a combination between chitosan, gelatin, and HA should induce the osteogenic differentiation due to two major aspects, namely the high osteoconduction and osteoinduction capacities of HA and the structure of chitosan, which is a polysaccharide derived from chitin that closely mimics the structure of human body polysaccharides, leading to an increased biocompatibility, antigenic and antimicrobial properties, and a suitable degradation rate [46]. Moreover, the reason for adding gelatin is based on the ability to form an RGD-like structure (Arginylglycylaspartic acid) that is very close in structure with the native bone ECM [47]. This, together with the addition of synthetic HA particles should be able to form a biomimetic scaffold. Therefore, the research group created electrospun nanofibers of chitosan and gelatin that were crosslinked with glutaraldehyde and then deposited the HA particles by a wet-chemical method, obtaining HA-chitosan-gelatin porous scaffolds. The scaffolds obtained were tested in contact with MG-63 cells and several parameters were evaluated, such as the biocompatibility and influence on cellular attachment and the proliferation at different time points. The importance of inorganic particles in scaffold composition was clearly remarked as they made a parallel testing on scaffolds with HA particles and the results confirmed the fact that the number of cells on the HA-chitosan-gelatin scaffold was significantly higher when compared to chitosan-gelatin scaffold. Additionally, after 5 days, there was observed a connection between cells, while on chitosan-gelatin the spreading rate was considerably low. Moreover, after 7 days, the cells covered the HA-chitosan-gelatin scaffold. Therefore, it was concluded that the biomimetic scaffolds which present porosity and imitate the bone ECM have promising results due to the existence of inorganic particles in their composition. HA particles were responsible for osteoconductivity, cell ingrowth, and bioactivity, which sustains the idea of guiding the cellular response by using proper materials for scaffold fabrication [46].

The theory of recreating the fibrous structure of ECM was also exploited by Januariyasa et al., who used carbonated-HA, chitosan, and polyvinyl alcohol to create a fibrous scaffold. Carbonated-HA was obtained by co-precipitation and subsequently added into the chitosan/polyvinyl alcohol solution. The resulted mixture was processed by the electrospinning equipment until the final carbonated-HA-chitosan-polyvinyl alcohol nanofibrous composite scaffolds were obtained. As expected, the protein adsorption was increased in the case of carbonated-HA scaffold, as well as its bioactivity after 7 days. Moreover, the inorganic content of the scaffold influenced, in a good manner, the cellular viability of the MC3T3E1 cell line and it enhanced osteoconduction when compared to chitosan-polyvinyl alcohol scaffolds [48]. Chitosan is not the only natural polysaccharide investigated together with HA, bacterial cellulose is gaining a lot of attention as well. Bacterial cellulose is a polysaccharide secreted by *Glucnacetobacter* microorganisms through oxidative fermentation processes and it has a great number of hydroxyl groups [OH\(^{-}\)] in its structure. This aspect is very important in terms of creating a friendly environment for proteins and cellular attachment and migration. Moreover, bacterial cellulose has a fibrous aspect, a good degradation rate, biocompatibility, and increased surface reactivity. Magnetic nanoparticles have been widely applied in the biomedical field and their inclusion in scaffolds for bone tissue engineering can be associated with their high susceptibility to be guided by magnetic fields directly to the targeted site. Owing to their ability to give an osteoinductive character to the scaffold with or without the influence of an external magnetic field, magnetic nanoparticles are suitable candidates for cellular signaling as well. As an example, magnetic bone scaffolds are taken into account by researchers due to their potential to activate the mechano-transduction process and to signalize the desired differentiation pathway. A study reported that the integration of magnetic nanoparticles in polycaprolactone polymeric scaffold promoted the osteogenic differentiation pathway of rat mesenchymal stem cells, and, additionally, in vivo investigations revealed the start of the angiogenesis process. Starting from these concepts, Torgobo et al. conducted an experiment where they developed composite scaffolds with the aim of creating a fibrous matrix of bacterial cellulose with
HA and magnetic nanoparticles dispersed in its structure. The physicochemical analysis concluded that a highly porous scaffold with mechanical properties close to those of the native trabecular bone was obtained. In vitro tests were conducted in contact with an MC3T3-E1 cell line and revealed non-cytotoxicity, high biocompatibility, and great adhesion and proliferation of the osteoblastic cell line \[49,50\]. Hence, it can be stated that porosity plays an important role in both mechanical and biological performances of a scaffold. From the biological point of view, it seems that cell anchorage is strongly influenced by pore size and architecture when compared to control samples. Currently, there is no doubt that porosity (nanopores, micropores, or macropores) has a positive impact in terms of promoting bone ingrowth and offering a biomimetic architecture to bone scaffolds. Even though porosity is not enough in terms of creating an ideal scaffold, it is a parameter that must be taken into account when developing bone matrices in order to achieve a native-like performance.

3.2. Three-Dimensional Printed HA-Based Scaffolds

Recently, additive manufacturing has gained a lot of attention in many industries, including the tissue engineering area \[51,52\]. Additive manufacturing offers the possibility of obtaining scaffolds that fit perfectly at the defect site, due to its ability to digitally recreate the damaged tissue before creating the scaffold. Even if there are multiple techniques that could be applied for creating tissue scaffolds, in the case of ceramic-based ones there are only two categories which are suitable for manufacturing: laser-based techniques and extrusion-based techniques. Intensive research has been made in order to manufacture HA-based structures able to induce osteogenesis and angiogenesis \[53\]. For example, Kim et al. stated that 3D-printed HA scaffolds present low strength and brittleness. In order to overcome this issue, they created a HA scaffold coated with polycaprolactone (used in order to increase the compressive strength) loaded with BMP-2 (bone morphogenic protein). BMP-2 is used in order to intensify osteoinduction and to accelerate bone healing. The samples were subjected to in vivo testing in rabbit calvarial defects and showed an improvement in terms of bone regeneration at 8 weeks after implantation when compared to uncoated scaffolds. However, the authors concluded that the use of BMP-2 is quite expensive for this type of clinical applications, since a large dose is needed for an effective response \[54\]. On the other hand, in a study conducted by Sun et al., a BMP-2 related peptide known as P28 was integrated in nano-HA scaffolds and showed higher adhesion rates and suitable proliferation percentages in cases of MC3T3-E1 cell lines when compared to controls (pure nanoHA) \[55\]. Millazo et al. stated in a comprehensive review on this topic that multiple HA-based composite scaffolds obtained via 3D printing processes were investigated over the years but most of them were based on polymeric matrices reinforced with HA particles of different sizes ranging from nanometers to micrometers \[53\]. Even if the results are promising for a broad range of polymers (e.g., chitosan, gelatin, silk, polycaprolactone, or collagen) from both physicochemical and biological points of view, the use of HA as a main and primary compound in 3D-printed scaffolds remains a challenge because of its mechanical properties which make it difficult to be processed by the available equipment. Moreover, the manufacturing of such products at an industrial scale is difficult especially due to the high costs involved in the design, production, and further in vitro, in vivo, and clinical set up testing.

3.3. Antimicrobial Scaffolds

As previously mentioned, there is one more aspect to be taken into account by scientists in order to overcome the infections that might appear at the defect site—finding an antimicrobial mixture which can reduce the bacterial adhesion and biofilm formation \[56,57\]. Until now, it was discovered that some materials can inhibit bacterial attachment both in vitro and in vivo \[58,59\]. For example, in a study conducted by Jiang in 2020, researchers developed chitosan-silk fibroin-based materials in multiple combinations: the first set of samples was made with polyethylene terephthalate, the second set with polyethylene terephthalate and HA, and the third set with polyethylene terephthalate and HA-silver nanoparticles. All materials were obtained by using the plasma-splash technique. These sets
of samples were subjected to in vitro and in vivo testing in rabbits. It was concluded that the best result in terms of cellular attachment was observed in the case of scaffolds with HA-silver in contact with mesenchymal stem cells. Moreover, osteoinduction was achieved by the HA-silver, a fact confirmed by evaluation after 14 days in contact with the same cellular type, when upregulation of multiple growth factors such as VEGF (promotes angiogenesis) and osteocalcin was validated [60]. Additionally, Zhang et al. proved that alginate could be used as a coating for porous HA scaffolds with the aim of immobilizing biomolecules and drugs (dexamethasone and stromal cell factor 1) in the form of microspheres. In vitro cell culture tests and in vivo evaluation on dogs confirmed multiple expected theories such as that: stromal cell factor 1 had an influence on cellular signaling and accelerated the recruitment of mesenchymal stem cells, the release of dexamethasone directed the differentiation of the same cellular line to osteogenic phenotype, and the results collected from canine models showed that newly-formed bone tissue was observed in the depth of the porous structure of the scaffolds [61]. Proper results were obtained in the case of human fetal osteoblasts as well by Fenice et al., who used polycaprolactone/zinc oxide scaffolds nanostructures loaded with HA particles. The choice of zinc oxide might be explained due to its corrosion rate which is very close to the one possessed by the natural bone tissue and because it is a fundamental element for the good functioning of a living human organism, being essential for the normal activity of about 3000 proteins. Moreover, it showed good antimicrobial properties and it acts as an osteoinductive stimulator [62]. The results for this type of scaffold showed that the ALP (Alkaline phosphatase) activity in contact with the above-mentioned cell phenotype was significantly higher—114 times more cellular viability was observed in the case of scaffolds containing HA and 6% zinc oxide when compared to simple polycaprolactone scaffolds [1,63]. Carbon dots were also investigated in combination with nanostructured HA and chitosan as scaffolds to evaluate the impact of carbon dots on ossification, reduction of bacterial attachment, and in treating osteosarcoma by photothermal treatment. In vitro studies on bone mesenchymal stem cells isolated from rats concluded that the addition of carbon dots and nanostructured HA is able to guide stem cell osteogenesis and a higher expression of ALP in comparison with simple chitosan-nanostructured HA scaffolds. After 2 weeks, collagen I and osteocalcin expression were higher for scaffolds containing carbon dots. In terms of their compatibility for tumor therapy, carbon dots exhibited photothermal effects in the presence of near-infrared spectrum radiations. Moreover, when subjected to this type of radiation their antimicrobial performance was present and the rate of *S. aureus* inhibition was about 99%, where for *E. coli*, the inhibition rate was 97% [64]. While antimicrobial properties do not play a direct role in the process of bone regeneration, prevention of infections at the damaged site is crucial. Infections impair the bone healing processes and lead to severe complications and, therefore, it is fundamentally important to consider this aspect when developing bone tissue engineering applications.

### 4. Conclusions and Further Perspectives

A lot of progress has been made in order to find proper treatments for diseases of the bones. The use of materials for manufacturing biomimetic approaches which might use the human body’s response to trauma and injury can lead to the development of a new generation of tissue engineered products. Scaffolds that exhibit dual functionality, such as osteoinduction and antimicrobial action, need to be taken into account in order to exclude the use of growth factors, antibiotics, and other substances which might increase the final cost for therapy and the negative impact on the patient recovery. Osteoinductivity, osteoconductivity, the role in mechano-transduction, and the composition of synthetic HA are just a few of the advantages that make HA a right choice for further research studies. Porosity proved to be one important parameter in the proper functionality of a scaffold, but to obtain a controlled pore size or network by conventional methods is still challenging. The approach which might help in getting a particular design and specific characteristics is represented by additive manufacturing, but issues related to costs and HA handling are still a challenge for researchers at the moment. However, the results obtained at this point are promising in terms of the biological response.
On the other hand, investigation is still needed to improve the dosages, the mixtures, or the techniques used for scaffold manufacturing. One example is in the case of silver-containing scaffolds, where the ratio of silver must be very carefully considered due to the side effects which may appear when administering a higher dose or, if at some point, the release of silver ions from the scaffold structure cannot be controlled.

Considering all the above-mentioned results, obtained in the last years by numerous research groups worldwide, there is a lot of room for improvements, but after a comprehensive in vitro, in vivo, and clinical evaluation of different materials and/or biomolecules combinations, there is a great potential to obtain the ideal product for bone regeneration.

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