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A Brief Review on Hydroxyapatite Nanoparticles Interactions with Biological Constituents

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Abstract

With the pursuit of new cancer therapies and more effective treatment to diseases in the last decades, nanotechnology has been an important ally for healthcare professionals and patients in critical clinical conditions. Nanomaterials offer an alternative way to deliver toxic chemotherapeutic drugs to specific biological tissues, specific cells or specific microbial beings, resulting in avoidance of strong side effects or resilience to effective drugs. Among these materials, stands out the hydroxyapatite nanoparticles, a ceramic class of calcium phosphates that present chemical and structural similarities with the mineral phase of the human skeleton's bone matrix, resulting in important biological features, such as biocompatibility, osteoconductive, osteoinduction and osteoaffinity, which led to a lot of scientific researches to apply these nanoparticles for bone diseases diagnosis and therapeutics. Due to the hydroxyapatite biological activities and due to the possibility to promote chemical and physical modifications in these nanoparticles, they can interact with biological cells or microorganisms in different ways, resulting in multiple potentialities to be explored such as apoptosis induction to cancerous cells, osteogenesis promotion, cellular proliferation, angiogenesis and tissue recovery, in addition to promote cell adhesion and cell uptake. Furthermore, chemical and physical modifications, such as surface functionalization, dopant inclusions and radiolabeling process, allow scientists to track the particle activities in biological environments. In the last decades of scientific productions, the literature brings together important data on how hydroxyapatite nanoparticles interact with biological tissues and such data are crucial for the development of more effective therapeutic and diagnostic agents. In the present review, we intend to compile scholarly information to explore the biological relations of

nanosized hydroxyapatite with the human cellular environment and the feasible modifications that may improve the theragnostic efficacy of these molecules.

Keywords

Biomaterials, Nano-Hydroxyapatite, Cell Interactions, Chemical Modifications, Tissue Engineering, Theranostics

1. Introduction

Nanotechnology is one strand of the science that studies the use, manipulations, production, and characterization of nanomaterials. The concept of nanotechnology is associated with a diverse manipulation of the nanomaterials through processes that involve the understanding and controlling of matter at the nanoscale, where unique phenomena enable new functional applications [1]. This concept brings to light the importance of controlling the chemical composition and structure of nanoparticles when these materials are designed for applications like cosmetics, construction, environmental, agriculture, fuels, dentistry, medicine and other applications with interest in social economics. In this context, scientists around the world have been gathering important data for biomedical applications of nanotechnology by researching many types of nanomaterials designed for diverse medical issues just like new alternatives of treatments and diagnosis of some pathologies. Such applications are generally based on the unique properties of the nanostructured materials that are determined by nanoparticles characteristics such as the very small size, shape, high surface area and surface reactivity, among other adjustable features [2] [3]. A biomaterial can be defined as "a substance that has been engineered to take form, which, alone or as a part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure" [4]. Considering this, some classes of nanomaterials can be applied as biomaterials, such as metals, polymers, composites and ceramics [5] [6].

Some classes of nanomaterials play crucial roles in the medical area acting as implants, prosthesis, as diagnostic and therapeutic agents or drug delivery systems, among other applications. Nanoparticles are composed of different materials, depending on the application for which it was intended [2]. Thus, nanoparticles can be synthesized from some material classes like metal, polymers and ceramics, or from a combination of these classes to constitute hybrid materials and nanocomposites with multifunctional features. Among the most common metallic materials used for synthesizing nanoparticles, zinc, gold, silver, iron, and titanium, have been exploited in scientific research, due to their mechanical, thermal and optoelectronic properties, in addition to their low reactivity and an acceptable biocompatibility [3] [7]. These features allow the use of metallic materials as biosensors, prosthesis, metallics structures for drugs, bio-imaging,

photothermal and radiation therapy [7] [8]. Scientists also investigate polymeric materials in biomedical research, such as polystyrene, polyesters, polyalkyl (meth)acrylates, polyurethanes and, polysaccharides, due to properties that differentiate them from other classes, such as biocompatibility, biodegradation, moldable crystallinity, generally low cost, and stability in biological medium [9] [10]. Some platforms are applied in medicine, such as liposomes, micelles, nanoemulsions, nanogels, nanosuspensions, capsules, and others, which are intended for drug delivery, catheters, sutures, cardio-vascular apparatus, bone cement, templates for vaccines or tissue scaffolds [10] [11]. Likewise, ceramic materials, as well as alumina, zirconia, bioglasses and calcium phosphates, have intrinsic properties like bioinert, biocompatibility, osteoconduction, osteoaffinity and osteoinduction, that make them valuable for medical applications, especially in tissue engineering, skeletal reconstruction, scaffolds, implants, and other therapeutic methods [8] [12].

The calcium phosphates stand out as bioceramics due to the close chemical composition to the skeletal tissues; these materials are generally classified by the Ca/P molar ratio [1]. Synthetic hydroxyapatite (HA) is the calcium phosphate known for the closest similarity with the mineral phase of the bone tissues. The molecular formula of this mineral is represented by $Ca_{10}(PO_4)_6(OH)_2$, which is mostly composed of calcium and phosphates with a molar ratio of Ca/P = 1.67, generally crystalized with a hexagonal arrangement. However, the different methods and parameters for synthesizing HA can lead to significant changes in the Ca/P molar ratio, crystallinity, surface area, particle size distribution and particle morphology [1]. These modifications directly influence properties like surface reactivity, adsorption, cellular adhesion, cellular uptake, and biocompatibility [13]. Features that define how hydroxyapatite interacts with biological constituents and determine the applications of this material for biomedical purposes [13] [14].

Ferreira-Ermita quotes the interaction between hydroxyapatite and eukaryotic cells, correlating some properties of both the materials and the hosts. The hydroxyapatite properties like form, composition, surface area, porosity, sterility, duration of contact, and degradation are checkpoints in biocompatibility [14]. Furthermore, some of these properties can awaken the immune system, promoting the activation of the defense system and favoring the biological integration of bone implants [5] [14] [15].

Some molecular mechanisms are fundamental for biological process like cell adhesion, adsorption, internalization, osteogenesis, angiogenesis, cell proliferation and differentiation, among others [14] [16] [17]. Proteins are generally the initiators of the interaction between nanoparticles and the biological environment [18]. According to WIJERATHNE and colleagues, hydroxyapatite nanoparticles play important hole in the colonization of microshapers by stimulating the synthesis of proteins that are responsible for adsorption of the nanoparticle to the tissues [19].

The induction of angiogenesis in microenvironments can be considered another

important aspect of hydroxyapatite for bone tissue engineering. The initial step for osteogenesis in bone rebuilding mechanisms is the angiogenesis, which is continued by cell proliferation and differentiation [20] [21] [22]. MALHOTRA and colleagues quote that angiogenesis is a result of the recolonization that hydroxyapatite causes in the tissue microenvironment. And that this is due to the increase in the number of cells in the region, which synthesize and release growth factors, stimulating the continuity of cell differentiation and proliferation [20] [22].

Besides its applications in bone reconstructions, hydroxyapatite nanoparticles have also been studied for tumor therapies [23] [24] [25]. However, this kind of application changes the material purpose and instead of inducing cell multiplication and differentiation, it should stimulate apoptosis and decrease cell proliferation [24]. The pathways for apoptosis activation rely on the adsorption and internalization of the nanoparticles in cancer cells, so that programmed death mechanisms are activated. In that sense, the control of hydroxyapatite nanoparticles characteristics is crucial for the desired application [26] [27].

The synthesis and manipulation of hydroxyapatite have been studied in several aspects of materials engineering, mainly associated with biological applications [28] [29] [30]. Despite the great number of published scientific researches about nanostructured hydroxyapatite for biological applications, we didn't find any work focusing on gathering information about hydroxyapatite nanoparticles interactions with organic tissues and cells. Considering this lack of information, this review aims to bring together the new findings about the biological mechanisms of interactions of hydroxyapatite nanoparticles and correlate such mechanisms to the inherent properties of hydroxyapatite.

2. Mechanisms of Interaction between Hydroxyapatite Nanoparticles and Biomicroenvironments

2.1. Pathways for Internalization and Localization of nHA

Many functionalities of nano-hydroxyapatite are based on the adhesion process and cellular internalization of these particles [31]. Some parameters are mentioned by authors so that the internalization occurs more efficiently being these mainly, size, morphology, crystallinity, and surface area [27] [32] [33]. SHI and colleagues demonstrated that hydroxyapatite size influences cell internalization considerably. They performed the experiment in umbilical cord mesenchymal cells, in order to use two distinct sizes of hydroxyapatite particles (20 nm and 80 nm), which were internalized by different molecular mechanisms. The 20 nm nHA particles were internalized by macropinocytosis while the 80 nm ones were internalized by endocytic pathways that are not very well known [27] [31]. Along with this information SHI *et al.* described the internalization process in two stages, the first is adhesion of particles to the cytoplasmic membrane and the second is their entry into cells, which will have the cytoplasmic vesicles and organelles as their destination. According to the authors, there were no changes in

the morphology of these cell vesicles.

Li and co-authors found different results in the intracellular location of nHA after being endocytosed and the cell type was a marked factor for this difference [34]. The authors used odontoblast-like cells as a reference. In this work, they identified nHA in the cell cytoplasm grouped or in phagocytic vacuoles. Similar to the results of Shi *et al.*, it was concluded that the size has great importance for endocytosis, in addition to adding that in their experiments they observed that the larger the size of the nano-hydroxyapatite, the more cytoplasmic vacuoles are caused, but nHAs were not internalized to the cell nucleus [27]. In the conclusion, the authors stated that it is not only the internalized concentration or the size that generates efficiency of the nanoparticles, but it also depends on other factors such as shape and crystallinity.

Furthermore, when nHAs are distributed in cytoplasmic vacuoles or in free aggregates in the cytosol, the cell type is a considerable interferer for localization and biodistribution of hydroxyapatite nanoparticles [27] [31] [33]. Another recurrent result mentioned in the literature is that the internalization time is inversely proportional to the size of the nanoparticle [27] [31] [35]. However, it was possible to conclude that the types and cell lines are variable in the purpose and fate of nHAs, as well as the size and shape of the particles [34].

According to Zhang and co-authors hydroxyapatite nanoparticles may have two distinct routes to get into the cells: endocytosis and phagocytosis [35]. In the first method, they cross the cell membrane and spread freely through the cytosol and get into organelles and nucleus. To achieve these results, the authors used different methodologies, modifying the sizes of the nanoparticles to approximately 40 nm, 73 nm and 100 nm, and the cells used were pre osteoblasts from rats, and not human cells like the cited by other authors, that can generate a relative difference when compared to other works. The second method of internalization is phagocytosis, in which the cell emits pseudopods and encompasses nHA, which are retained in endosomes, being subsequently transported to lysosomes, and finally dissolved within the vesicle. It is worth highlighting the influence of surface functionalization of the HA in the degree of internalization of these nanoparticles after chemical modification.

ZHANG and colleagues used folic acid to increase cell uptake, and according to their results these functionalized nanoparticles significantly increase the uptake of nHAs [25]. The cell uptake of functionalized nanoparticles can be favored by a biological factor called Enhanced Permeabilization and Retention effect (EPR), which has been explored by several researchers for target therapeutics [25] [36] [37]. This effect can be conceptualized as a higher permeabilization caused by a higher fenestration between bloody vessel cells in tumor vascularity that leads to a high metabolic rate of cancer cells which affects the greatest need for nutrients as energy sources for cell proliferation and exacerbated growth [36]. Folic acid is an important nutrient for cancer cells and, due to the high metabolic rate, cancer cells present overexpression of folic acid receptors in their membrane to activate internalization mechanisms of this molecule, allowing the

using of folic acid as a targeting molecule [38].

Membrane surface charge has also been discussed in the literature, in which positively charged particles present greater internalization in cells, thus increasing the number of cytoplasmic vacuoles in the cell interior. Chen *et al.* performed tests with MC3T3-E1 osteoblasts, in which it was found that when these nanoparticles were treated and with positive surface charges, it generated greater internalization flow, and in samples of untreated and negatively charged nHAs the internalization ratio dropped considerably, both experiments used nHAs with similar shapes and sizes. So the surface charge of the material is also an important factor in the internalization of these particles [39].

The mechanisms of internalization depend on adjustable characteristics of materials and inherent features of the organic tissues and cells. Considering these issues, hydroxyapatite nanoparticles characteristics must be tuned considering the kind of tissue in which the material intended to be used as a therapeutic or diagnostic agent.

2.2. Cell Adhesion and Adsorption Associated with Nano-Hydroxyapatite

The association between hydroxyapatite or nano-hydroxyapatite and bone reconstruction process is recurrent in many scientific publications [40] [41]. For this process to take shape, the adsorption and adherence of nanoparticles to cellular proteins are key points [42] [43]. Wijerathne and coauthors [19] demonstrated that the introduction of nano-hydroxyapatite in the composition of poly (lactic acid) microspheres increases the adhesion rate of bovine serum albumin to this particles, resulting in improved rat mesenchymal stem cells adhesion and proliferation, acting as a facilitator agent for osteogenic differentiation [44] [45] [46]. Lin and coauthors [47] investigated how the structural properties of nano-hydroxyapatite influence the induction of cell adhesion. This work shows that through controllable nanotopography of nHA bioceramics, it is possible to enhance adhesion, spreading and osteogenic differentiation of osteoblasts by accelerating early cell responses in the cell attachment process such as protein adsorption.

In tissue engineering, scaffolds are prepared for bone tissue reconstruction and one of the expected results is the wide production of proteins and peptides by the cells through chemical interaction with bioparticles, generating the elevation of second cellular messengers that send the necessary information to the cell nucleus, which increases protein synthesis [42] [48]. With this increase, nanoparticles tend to improve the degree of adhesion with the cellular environment [42] [49]. According to Costa *et al.*, "the focal adhesion of proteins also mediate signal transduction and subsequent changes in gene expression initiated by binding to the extracellular matrix or biomaterial surface" [50] [51]. The increase in gene expression results in an increase in protein synthesis along with the induction of osteogenesis, with the beginning of bone restructuring by cell colonization by osteoblasts, which synthesize integrins thus increasing the capacity for adsorption and adhesion of cells [42] [47] [48]. The internalization of

the nanoparticles generates losses of cell adhesions in cancerous cells, resulting in loss of cell to cell binding that makes the cells undergo apoptosis [48] or promotes structural changes in the cells which affect the adsorption and activation of membrane receptors [43] [52].

Thus, the important factor that will define the adhesion gain in healthy bone cells is the bioactivity capacity of hydroxyapatite nanoparticles when in contact with the cell membrane, which activates signal transduction pathways increasing the synthesis and release of growth factors and proteins in general [18] [19] [48]. On the other hand, the reduction of adhesion in initial cancer cells occurs through the increase in the internalization of nanoparticles, which directly interferes in the nucleus and in the cellular genetic material, promoting a reduction in the synthesis of growth factors and proteins for the continuity of metastatic processes [53].

2.3. nHAs-Induced Cell Proliferation and Differentiation

Cell proliferation and differentiation are common processes in the formation and regeneration of tissues, especially bones, and these are fundamental in tissue engineering. Some nanocomposites, such as based-hydroxyapatite materials, have the potential to induce cell maturation and proliferation, in addition to serving as a structure for tissue development [54]. According to Yang and colleagues, nano-hydroxyapatite has enough bioactive properties to support the formation and colonization of new tissue [55]. In their work, the Ca^{2+} and pH dependent potential of nHA to act as a stimulating agent for osteogenic cells differentiation were evidenced through measurements of bone expression markers, like osteopontin, osteocalcin, runt-related transcription factor 2, and alkaline phosphatases, when these nanoparticles were incubated in the presence of human mesenchymal cells and comparing these results with the control group The use of nanostructures for grafts has shown enhanced potential for cell proliferation, as the decreasing in particle size increases the contact surface between material and cells in the cellular microenvironment, which provides more adsorption sites for the interaction of bioactive molecules [47]. Some in vitro studies confirmed that nano/micro-structured surfaces could enhance the osteogenic differentiation of mesenchymal cells and osteoblasts even in the absence of growth factors, subsequently leading to the acceleration of osseointegration and bone regeneration [47] [48]. The mechanisms behind this increasing are associated with the similarity of the natural structure that the nanomaterial offers to the microenvironment. In this way, the cells would have a matrix like natural which promotes cell proliferation and differentiation, which increases proteins and the number of resident tissue cells [47] [54] [56].

Han and coauthors compared the inhibition effect in cell proliferation that nanoparticles can cause in cancer cells and normal cells [57]. For this study, the authors used three distinct types of normal cells (pulmonary fibroblasts, keratinocytes, and hepatocytes) and three types of cancer cells (osteosarcoma, gastric and hepatocellular carcinoma cells). They concluded that nano-hydroxyapatite

inhibits cell proliferation in both healthy and tumor cells but in different proportions and in a not-dose-dependent manner, stating that the variation between the cell types is fundamental to predict the therapeutic efficiency of the nanoparticles. The authors quantified the time-dependent inhibition effect in the cancerous cell lineages, which variated from 54.3% - 65.0%, 60.6% - 75.8%, and 76.3% - 88.0% for the gastric carcinoma (MGC-803), osteosarcoma (Os-732) and hepatocellular carcinoma (Bel-7402) types, respectively [47] [57]. Han and coworkers proposed a molecular mechanism, based on a decreasing synthesis of essential proteins for cell maturation, proliferation, and differentiation, where the nanoparticles interfere in the translation process of cancerous and normal cells in different proportions. The authors also proposed that the tumor cells were more damaged due to the EPR effect and due to the higher internalized concentration of nano-hydroxyapatite when compared to normal cells. On the other hand, works carried out by Wang and colleagues showed that hydroxyapatite nanoparticles act in a dose-dependent manner in biological media up to a certain concentration, when large doses may not perform a proportional response, since there is a threshold for incorporation of particles into cells and tissues, influencing the final efficiency of the nanoparticles [21] [57].

However, the main mechanisms that promote the differentiation and proliferation of bone cells are associated with the nanotopographic similarity that hydroxyapatite nanoparticles present when inserted in bone microenvironments [16] [58]. In addition to the material's bioactivity, it also induces increased synthesis of proteins and growth factors that actively participate in the tissue reconstitution process [59]

2.4. nHAs-Induced Angiogenesis and Vasculogenesis

When the restructuring of a tissue is taken into account, means of obtaining nutrients and oxygen is necessary for the new cells that are going to settle and begin processes of proliferation, maturation and cell differentiation. It is known that cells are able to energetically maintain themselves through the bloodstream, which dispose of large contents of substances and gases that are necessary for their metabolism and allow the regulation of the metabolites excretes [20] [60]. Thus, as well as the other factors mentioned, the induction of angiogenesis is one of the fundamental processes in the efficiency of the formation of a new tissue [17]. As the bone tissue is remarkably favored with the presence of ideal concentrations of hydroxyapatite, according to some authors, the bone recovery process involves several steps including the formation of new blood vessels, which will serve to nourish the new developing tissue [16] [60].

Blood vessels are formed by means of stimuli, which depend on certain steps for the vascularization of a given region to be viable and efficient, where three processes can stand out: vasculogenesis, angiogenesis and arteriogenesis [16]. The stages of angiogenesis are relatively different from that of vasculogenesis, as the former is a process more dependent on the embryological profile [61]. Initially, angiogenesis develops an increase in vascular permeability, resulting in

indirect protein excretion and degradation of the basement membrane with remodeling of the extracellular matrix that causes the release of growth factors that triggers a sequence of processes resulting in the angiogenesis of a tissue region [16].

The intrinsic characteristics of nanobiomaterials, as pore architecture, surface nanotopology and material stiffness, can favor the angiogenesis by enhancing the stimuli of recolonization components of the cellular matrix in the process of tissue reconstruction [58]. Nano-hydroxyapatite itself presents regenerative properties. However, some physical and chemical modifications like doping processes can be induced in this material to modulate and increase the angiogenic and apoptotic effects. [62] [63].

The concept of doping is associated with the introduction of ions, generally metallic, in the atomic structural lattice of materials [64]. In ceramic materials, specifically hydroxyapatite, dopant ions generate small degrees of instability in their crystallites due to the structural disorder and this effect may vary according to the kind and number of inserted ions, that should be planned accordingly to the purpose of application Priya *et al.* synthesized nickel-doped nano-hydroxyapatite sizing from 14 nm to 18 nm with non-toxic concentrations of the metallic ion, showing slight changes in morphology due to the doping process. The authors used BSA as a model to test the protein adsorption potential and they concluded that the surface area and the distribution of calcium, nickel and phosphate charges directly influence the protein adsorption. Researches stated that these factors influence the cell differentiation process probably due to the increase of proteins and growth factors such as Runx2, osterix, osteocalcin and, mainly, VEGF (Vascular endothelial growth factor), which had significantly proportions increased when compared to pure hydroxyapatite particle. Bose and coauthors suggest mechanisms of action for some dopant ions, such as magnesium (II), copper (I), cobalt (II), boron (III) and silicon (IV), to explain the osteogenesis or angiogenesis increasing (**Table 1**) [62].

Moreover, dopants like copper ions can regulate the expression of angiogenesis-related factors, such as vascular endothelial growth factors (VEGF) and hypoxia inductive factor (HIF), promoting the proliferation of endothelial cells and osteoblast differentiation [59]. Elrayah and coworkers developed hydroxyapatite scaffolds with controlled micro/nano-structured surfaces by varying copper ion content in the hydrothermal process, changing significantly from sphere-like to flower-like with the increase in Cu^{2+} concentration. In vitro assay with endothelial cell cultures showed significantly enhanced cell proliferation in the presence of flower-like morphology scaffolds compared with other morphologies. Likewise, in vivo tests with rabbits demonstrated that the flower-like surface scaffolds promoted more angiogenesis compared with the control scaffold [65].

As well as cell proliferation and differentiation, angiogenesis and vasculogenesis are induced from the nanotopological similarity that hydroxyapatite particles present in the medium, associated with the increase of cells residing in the tissue, the need to increase nutrition pathways is important, and these new cells have

Table 1. Bioapplications and functionalities of metal ions in tissue microenvironments.

Metallic ion	Role	Mechanisms of action
Li (I)	Osteogenesis	Inhibit GSK3, which is a negative regulator of the Wnt signaling pathway. Activates b-catenin-mediated T cell factor (TCF)-dependent transcription during bone and cartilage fracture healing. b-catenin is known for its central role as signaling mediator in the canonical Wnt signaling pathway.
Zn (II)	Osteogenesis	In the cellular microenvironment, zinc is thought to stop the osteoclastic resorption process and stimulate the osteoblastic bone building process.
Mg (II)	Angiogenesis	Magnesium induces nitric oxide production in endothelial cells which is essentially the same mechanism that VEGF uses to induce angiogenesis.
Sr (II)	Osteogenesis	Strontium stimulates bone formation by a dual mode of action: a stimulatory role on bone-forming osteoblast cells and an inhibitory role on bone resorbing osteoclast cells. It activates CaSR and downstream signaling pathways. This promotes osteoblast proliferation, differentiation, and survival; at the same time, it induces apoptosis in osteoclast cells resulting in decreased bone resorption. Activation of the CaSR in osteoblasts simultaneously increases OPG production and decreases RANKL expression. OPG is a protein that inhibits RANKL-induced osteoclastogenesis by operating as a decoy receptor for RANKL.
Cu (I)	Angiogenesis	Copper-induced angiogenesis is probably caused by the upregulation of VEGF expression. Copper-induced toxicity: at high concentration, copper can generate ROS in the presence of superoxide radical anions (O_2). These ROS induce oxidative damage to cells through DNA strand breaks and oxidation of bases.
Co (II)	Angiogenesis	It is believed that Co (II) ions induce hypoxia on the cellular level by stabilizing HIF-1a. Cells compensate for this hypoxic environment by expressing genes (such as VEGF and EPO) that promote neovascularization and angiogenesis. Cobalt-induced toxicity: like copper, cobalt also causes oxidative damage to cells by ROS. Increased soluble Co (II) ion levels might cause serious adverse reactions to the surrounding tissues as well as systemic toxicity. Co (II) ions can activate and increase bone resorbing osteoclast cell differentiation resulting in osteolysis aseptic implant loosening.
B (III)	Osteogenesis/ angiogenesis	Thought to play a role in the upregulation of TGF-b and VEGF.
Mn (II)/(III)	Osteogenesis	Thought to have implications in the PTH signaling pathway, a key regulator of calcium. MnSOD is believed to neutralize the formation of ROS, which contribute to increased osteoclastogenesis and decreased osteoblastogenesis.
Si (IV)	Osteogenesis/ angiogenesis	Has been shown to induce angiogenesis by upregulating NOS leading to increased VEGF production. Osteogenic mechanisms are not well understood, but Si (IV) has been shown to play a vital role in the mineralization process.

Source: Bose *et al.*, 2013.

increased the synthesis of growth factors thus inducing angiogenesis in the region [16] [58]. Furthermore, in the literature, metal ion doping is described as a way to increase the efficiency of the biomaterial in the tissue vascularization process [66] [67].

2.5. Selective Cytotoxicity of nHAs

The cytotoxicity of a nanomaterial depends on some factors inherent to the host

or the material, such as size, composition, physicochemical modifications, surface area, porosity, sterility, concentrations or tissue type, genetics of the individual, among others [14]. These properties are directly related to the immunological responses which these biomaterials promote in the cellular environment and the modulation of these factors is responsible for the circulation time and the clearance of a nanomaterial [13] [14] [68].

The human body is provided with exogenous or endogenous metabolic mechanisms for internalization of substances as methods of defense, synthesis, energy obtaining, and electrolyte balance [69]. Each tissue and cell type reacts differently when exposed to exogenous substances. Hydroxyapatite nanoparticles, despite their similarity with the inorganic phase of bone tissues, present chemical and physical variations that make some cell types treat them as invading agents, other cell types as tissue components or, yet, as a source of obtaining metabolites and energy [69]. The interaction and absorption of these nanoparticles can generate problems of biocompatibility since they can cause deregulations in electrolytic balances, altering metabolic pathways of cells or induce them to apoptosis [32]. There are two main effects that particles can generate to the microenvironment: 1) the elimination or reduction of a given cell type and 2) the increasing of cell proliferation and regeneration of tissues [54] [70].

Considering that orthopedics and orthodontics are the areas that most seek applications of nano-hydroxyapatite, there is an emergency need to evaluate the toxicity of nHAs in bone tissues. It was demonstrated by Ocampo and teammates that human osteoblasts exhibited cell viability above 90% during in vitro assay with hybrid polysaccharide-hydroxyapatite nanorods which were considered non-toxic to humans [71]. Chen *et al.* studied the biocompatibility of bone cells (osteoblasts-MC3T3-E1) when treated with hydroxyapatite nanoparticles, and obtained as a result that this material has high cell viability, and influencing these cells to produce higher concentrations of growth factors and cell differentiation in addition to inhibits cell membrane damage. According to them, this is due to the internalization of these particles, but they report that further studies are needed [39]. Another study, by Cipreste *et al.* also revealed high indices of biocompatibility in fibroblasts at concentrations of hydroxyapatite nanorods up to 100 μg/mL, and which in conclusion highlighted that these particles functionalized with folate can be used in bone reconstruction treatments, due to the inducing characteristic of the material, favoring proliferation and tissue differentiation [37].

Some features of an in vitro assay designed can be determinant for the cell viability [32] [69] [72]. For example, Geetha and coauthors tested the cell viability of fibroblasts (L929) to access the cytotoxicity of nHAs and they concluded that features like pH, concentration and the size of hydroxyapatite nanoparticles are decisive for outcomes, indicating that up to 600 μg/mL the results show 80% cell viability with 50 nm nano-hydroxyapatite [68]. Similarly, Szymonowicz and partners performed the tests with the same fibroblast lineage but variating the size of the nanoparticles which showed cell viability inversely proportional to

their length [73]. Furthermore, the variation of cell types can also be considered a striking factor for ultimate cell viability [14] [62]. Liu and Sun studied the cytotoxicity of elongated hydroxyapatite nanoparticles at concentrations greater than 100 μg/mL in umbilical cord endothelial cells that showed significant toxicity, unlike the results found by Geetha *et al.* in fibroblasts [13] [68]. A question raised by these authors is that there is a threshold in the dose applied to cells whose value above this threshold can induce them to a cell death process.

Rao and collaborators studied the interaction of renal epithelial cells with hydroxyapatite nanoparticles and described that internalized nHAs concentrate in lysosomal vesicles [33]. In this work, the authors focused on the formation of kidney stones and the toxicity that their accumulation generates when they come into contact with the renal epithelial cells. As a result, hydroxyapatite can lead these cells to apoptosis. Therefore, an important fact is that nHAs in lysosomes induce the generation of reactive oxygen species that triggers a programmed cell death as consequence, which is an important apoptotic mechanism in cancer cells that can be exploited for cancer therapies [33] [68] [72].

The ability of hydroxyapatite to produce free radicals and reactive oxygen species (ROS) causes oxidative stress in the cells and can induce pathways of cell death. However, Turkez and colleagues demonstrated that blood cells exposed for 72 hours with nanoparticles with diameters ranging from 10 to 50 nm up to 150 ppm cannot produce enough toxic elements to induce an increase of free radicals and ROS [72]. This is an important finding because some therapeutic applications of nHA are through blood distribution or in regions heavily irrigated by blood, which is one of the problems involving the biocompatibility of the material [74]. Concentrations above 150 ppm can damage the genetic material of cells due to oxidative stress, which can be of interest for the specific treatment of cancer [72], considering the that nanoparticles tend to accumulate selectively in cancer sites due to passive or active targeting through EPR effect or functionalized ligands on nanoparticles surfaces, respectively [36].

The selective toxicity of hydroxyapatite nanoparticles for cancer cells can also be evidenced in means of cell type. In the toxicity experiments conducted by Tang *et al.*, 3 types of cancer cells were used: gastric cancer cells (MGC80-3), cervical adenocarcinoma (HeLa) and hepatocarcinoma (HepG2), in addition to a healthy liver cell line (L-02) [70]. The authors tested nHAs with 50 × 20 nm with concentrations in the range of 62 to 1000 μg/mL, for 24, 48, and 72 hours of exposure. The results show that the HepG2 and HeLa strain needed concentration of 1000 μg/mL for 72 hrs. to reach the threshold of growth inhibition. MGC80-3 cells, on the other hand, required 500 to 750 μg/mL for 48 hrs, or 72 hrs. to reach the maximum limit of cell inhibition, unlike normal liver cells, which, even at concentrations of 1000 μm/mL, did not show changes in the index reduction in proliferation. The authors proposed that the intracellular localization and distribution occurs differently when compared to normal cells due to the metabolic changes that cancer cells present [70]. In this way, the unregulated intracellular ionic variation generates the release of free radicals that dam-

age the genetic material of neoplastic cells. However, it seems that neoplastic cells suffer more from nanoparticles, not because of the concentration of internalized molecules, but due to the metabolic variations they present [23] [48] [70].

The cytotoxicity of hydroxyapatite nanoparticles is generally associated with electrolyte and metabolic imbalance, which are characteristic of cancer cells. In this way, these cells internalize large concentrations of nanoparticles that damage the cell, inducing it to death. However, most of the healthy cells mentioned in the consulted literature showed an acceptable degree of cytotoxicity in human cells, due to little internalization of these nanoparticles, thus obtaining high biocompatibility values. Furthermore, an interferent is a cellular type, which may culminate in different results upon treatment with nHAs [48] [70].

Cellular apoptosis is conceptualized as a programmed cell death which mechanism can be triggered as physiological or pathological process for senescence, development or defense, acting as an important principle in the control of cell populations and the renewal of tissues (Obeng *et al.* 2021). Apoptosis is associated with the cytotoxicity that the nanoparticles cause to the microenvironment and may be swayed by the amount of uptake particles, thus the rate of apoptosis depends on nanomaterial characteristics such as size, shape, surface charge, concentration and time of interaction [75] [76]. Cancerous cells can capture high concentrations of hydroxyapatite nanoparticles due to the EPR effect, resulting in increased free radical synthesis by cell organelles that leads the cancerous cells to death [77] [78].

According to Guo and coauthors, a mutation that influences the signaling pathway of nuclear factors (NF-kb) prevents cancerous cells from starting the apoptosis process, which results in a high population that leads to the development of tumors [24]. However, they stated that nano hydroxyapatite blocks this pathway by generation of free radicals, making these cells undergo apoptosis or being arrested in phases G2/M of the cell cycle, reducing the proliferative capacity of the tumor anyway. Moreover, the crystallinity degree of hydroxyapatite nanoparticles influence the dissociation of calcium ions in the cellular environment which interferes in the number of apoptotic bodies and in the number of cells arrested in cell cycle; the greater the disorder of the material, more easily it will release ions in the medium which positively influences the rate of death of tumor cells, differently what happens to normal cells that present greater control over their metabolism and, as a consequence, regulating better the inflow and outflow of ions what result in no effect [35] [76] [79] [80].

Cellular apoptosis has been described as a consequence, mainly, in treatments of neoplastic cells, with nHAs, due to the metabolic and electrolytic lack of control of the cells, which increases the degree of internalization of the nanoparticles, inducing the exacerbated synthesis of free radicals, which are released through organelles and attack important components of the cell, such as cytoskeletal proteins or genetic material. In this way, it triggers the process of programmed cell death due to damage caused by free radicals [24] [78] [81].

3. Conclusion

The present review distinguishes two main applications for hydroxyapatite nanoparticles, the reconstruction of bone tissue and the aspect of elimination of cancer cells. The efficiency of these materials for the former application is associated with the material's interaction with cell membranes, which induce an increase in the production of proteins and growth factors that helps in the reconstruction, especially of bone tissues. The inherent properties of the material that will directly influence this factor are 1) the size of the particles that dictate the contact time with the cells; 2) the crystallinity that influences the dissociation of ions resulting in the increasing in signal transduction; and 3) the particles shape related to the surface contact area. The application of nHA for cancer treatments is related to the cellular apoptosis induction, which is associated with a higher concentration of internalized nanoparticles that cause structural damage to the cellular genetic material when these nanoparticles stay inside the cell in large proportions. The efficiency for this application is also related to the particles size, in which the smaller particles get faster into the cells; the particles morphology, in which more compact structures are more easily internalized, and the crystallinity degree that defines the ionic dissociation rate and the formation of free radicals which positively influence the induction of cancer cells to the apoptotic pathway. Another issue elucidated is that the surface charge of the particle can considerably influence the cellular internalization process, in which positively charged particles would enter more easily and in higher concentrations into cells, thus increasing the effectiveness of the treatment. The literature brings important data for researchers that tend to investigate how to improve hydroxyapatite nanoparticles properties aiming applications in tissue engineering and cancer treatment and the present review can be useful for these researches due to the reunion of many relevant findings on hydroxyapatite interactions with biological constituents and the tunning properties of this material that influence these interactions.

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Conflicts of Interest

All the authors declare that they have no known competing for financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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