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Overview of Hydroxyapatite–Graphene Nanoplatelets Composite as Bone Graft Substitute: Mechanical Behavior and In-vitro Biofunctionality

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ABSTRACT
Hydroxyapatite (HA) and related materials have been frequently studied as ceramic-based bone graft materials due to their outstanding biocompatibility and osteoconductivity. Since the bones are the load supporting parts of a vertebrate, they must have good fracture toughness ($K_C$) to avoid fracture at high loading during limb movements. However, the main shortcomings of HA are the poor fracture toughness and brittleness. The mechanical properties of HA need to be improved for orthopedic applications, therefore it is often fabricated with other materials into a composite. This article focuses on the effect of carbon nanostructures (CNSs) especially graphene nanoplatelets (GNPs) on the mechanical, physicochemical properties and in-vitro bio-functional performances of HA. We provide an overview on the preparation and characterization of the HA–GNPs composites. To conclude, the challenges in the fabrication of multi-substituted HA–GNPs composites and future outlooks in the biomedical domain are discussed.

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KEYWORDS
Hydroxyapatite; carbon nanostructures; composite; ion exchange; mechanical properties; in-vitro bioactivity

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1. Introduction

The earliest bone-grafting procedure reported in the literature was by Job van Meekren, a Dutch surgeon in 1668. The efforts by Lindholm and Urist in 1980 incorporated bone marrow into the bone matrix, resulting in new bone formation. Later, a successful percutaneous bone marrow injection for tibial non-unions was reported by Connolly and Shindell in 1986. This was followed by the successful treatment of tibial nonunions with either the use of a Lottes nail or a cast in 1991. Nowadays, people are paying more attention to rehabilitation and medical care due to the improvement in living conditions. Injuries to human hard tissue system cause more than a million surgeries each year. Therefore, the demand for biomaterials to facilitate bone recuperation from infection, tumors, damage, and osteoporosis/osteomalacia, due to aging, has increased recently at a rate of 18–20% per year. This suggests that the quality and quantity of bone graft substitutes needed to be enhanced, where the clinical success of a bone graft substitute is strongly dependent on its biological and mechanical properties. It should be mentioned here that the term bone graft substitute refers to a range of materials that may have diverse effects on the bone rehabilitation process. As shown in Figure 1, bone grafts are generally classified based on the type of materials:

(i) Allograft-based bone grafts. This includes allograft bone, which can be used alone or combined with other materials, such as Grafton and OrthoBlast.

(ii) Factor-based bone grafts. These possess recombinant and natural growth factors, such as bone morphogenetic protein (BMP), transforming growth factor-beta (TGF-beta), fibroblast growth factors (FGF), and platelet-derived growth factor (PDGF).

(iii) Cell-based bone grafts. This method uses cells for new tissue generation, either used alone or seeded into a matrix such as mesenchymal stem cells.

(iv) Ceramic-based bone grafts. These involve bioactive glass, calcium phosphate, and calcium sulphate synthetic substitutes, used alone or combined with OsteoGraf, ProOsteon, or OsteoSet.

(v) Polymer-based bone grafts. These utilize degradable and non-degradable polymers, used alone or combined with other materials.

From a biological mechanism standpoint, bone graft substitutes can be categorized as osteogenic, osteoconductive or osteoinductive, as demonstrated in Figure 2. Osteoinduction was first introduced after the discovery of bone induction principle by Urist in the 1960s. One of the distinctive features of the mammalian endo-skeleton is the regeneration ability, which involves a series of occurrences similar to embryological development. In the early development of a repair process, the undifferentiated mesenchymal cells differentiate due to the presence of some external stimulus is also known as osteoinduction, or can be described as “a process that supports the mitogenesis of undifferentiated mesenchymal cells with the ability to form a new bone.” Consequently, any material which exhibits this process is considered as osteoinductive.

On the other hand, osteoconduction is a process where the bone graft material becomes a scaffold for new bone generation, which is initiated by the natural bone. The grafted osteoblasts utilize the bone graft for the expansion and growth of new bone. Osteogenesis is a process where the osteoblasts derived from the bone graft material play an important role in the generation of new bone material. A high percentage of bone graft substitutes presently available in the market are ceramic-based materials, either used alone or in combination with another material (Table 1).

This class of bone grafts involves bioactive glass, calcium sulfate and phosphates. Among the diverse forms of calcium phosphates (CPs), tricalcium phosphate \( \text{Ca}_3(\text{PO}_4)_2 \) (TCP), and hydroxyl apatites (HA) \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \) have gathered much interest due to their outstanding biocompatibility and osteoconductivity. CPs are also osseointegrative and in some cases, osteoinductive. Accordingly, these bioceramics have been widely used in medical applications especially as ceramic-based bone graft materials. However, the main disadvantage of HA is the poor wear resistance and fracture toughness. The fracture toughness of HA \( (1 \text{ MPa.m}^{0.5}) \) is lower than the lowest value of the cortical
bone (2 MPa.m$^{0.5}$). The poor fracture toughness also gives rise to lower wear resistance, in view of the fact that wear volume loss in ceramics is directly influenced by the fracture toughness. The bones are the load supporting parts of a vertebrate, therefore must possess sufficient fracture toughness ($K_{IC}$) to avoid fracture and cracking, upon high cyclic loading during movements. Thus, the fracture toughness of HA needs to be enhanced for orthopedic applications. Additionally, they have brittle features and frequently need higher temperatures for scaffold formation, therefore are often combined with other materials as composites.

An ideal reinforcement material must improve the mechanical properties of HA significantly, even at low percentages, without jeopardizing the biological properties of the composite structure. Besides, it is essential that the reinforcing agent possesses an excellent combination of elastic modulus and strength, so that the fracture toughness of HA increases significantly with small additions of the reinforcement. Accordingly, studies on the use of carbon nanostructures (CNSs) especially graphene in orthopedic applications have grown steadily in recent years. Graphene is endowed with excellent mechanical properties (e.g., elastic modulus $E$) with the presence of the sp$^2$ carbon network. The modulus of elasticity of a single-layer graphene is theoretically around 1.02 TPa. The fracture strength of a defect free flat-shaped graphene sheet is experimentally validated as 42 N m$^{-1}$. 

![Figure 1. Classification of bone grafts with respect to the material groups.](image1)

![Figure 2. Properties of bone graft substitutes.](image2)
Table 1. Some ceramic-based bone graft substitutes. (© Taylor & Francis Group. Reprinted with permission from Laurencin et al.5 Permission to reuse must be obtained from the rightsholder.)

<table>
<thead>
<tr>
<th>Product name</th>
<th>Company</th>
<th>Subgroup</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteograft®*</td>
<td>Ceramed Dental, Inc.</td>
<td>Ceramic-based</td>
<td>Synthetic HA particles and blocks</td>
</tr>
<tr>
<td>Norian SRS®</td>
<td>Synthes, Inc.</td>
<td>Ceramic-based</td>
<td>Calcium phosphate cement</td>
</tr>
<tr>
<td>ProOsteon®</td>
<td>Interpore Cross Intl., Inc.</td>
<td>Ceramic-based</td>
<td>Coraline HA</td>
</tr>
<tr>
<td>Osteosite®</td>
<td>Wright Medical, Inc.</td>
<td>Ceramic-based</td>
<td>Calcium sulfate</td>
</tr>
<tr>
<td>Stimulan®</td>
<td>Encore Orthop., Inc.</td>
<td>Ceramic-based</td>
<td>Calcium sulfate</td>
</tr>
<tr>
<td>Hapset®</td>
<td>Lifecore Biomed, Inc.</td>
<td>Ceramic-based</td>
<td>Calcium sulfate</td>
</tr>
<tr>
<td>Collagraft®</td>
<td>Zimmer, Inc.</td>
<td>Ceramic-based</td>
<td>TCP, HA, collagen</td>
</tr>
<tr>
<td>Calcitite®</td>
<td>Centerpulse, Inc.</td>
<td>Ceramic-based</td>
<td>HA</td>
</tr>
<tr>
<td>Osteogen®</td>
<td>Park Dental Res., Corp.</td>
<td>Ceramic-based</td>
<td>Calcium-deficient HA</td>
</tr>
<tr>
<td>BoneSource®</td>
<td>Howmedica Leibinger, Inc.</td>
<td>Ceramic-based</td>
<td>Calcium phosphate putty</td>
</tr>
<tr>
<td>α-BSM</td>
<td>E-Tex, Inc.</td>
<td>Ceramic-based</td>
<td>Calcium phosphate cement</td>
</tr>
<tr>
<td>Healos®</td>
<td>Orquest, Inc.</td>
<td>Ceramic-, polymer-based</td>
<td>Collagen and HA</td>
</tr>
<tr>
<td>Vitoss®</td>
<td>Orthovita, Inc.</td>
<td>Ceramic-based</td>
<td>β-TCP</td>
</tr>
<tr>
<td>Bio-Oss®</td>
<td>Geistlich Biomaterials, Inc.</td>
<td>Ceramic-based</td>
<td>Bovine HA</td>
</tr>
<tr>
<td>Novabone®</td>
<td>US Biomaterials, Inc.</td>
<td>Ceramic-based</td>
<td>Bioactive glass</td>
</tr>
<tr>
<td>Perioglas®</td>
<td>US Biomaterials, Inc.</td>
<td>Ceramic-based</td>
<td>Bioactive glass</td>
</tr>
<tr>
<td>BioGran®</td>
<td>Orthovita, Inc.</td>
<td>Ceramic-based</td>
<td>Bioactive glass</td>
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However, the broad application of graphene is frequently restricted by its production, storage and processing. So, the stabilization and modification of graphene have attracted considerable attention.22 Graphene oxide (GO) is a single layer of graphene with epoxide, carbonyl, carboxyl, and hydroxyl functional groups. On the other hand, the reduction of GO to reduced graphene oxide (rGO) has a huge impact on the quality of the final product; this will determine whether the structure and properties of rGO is comparable to pristine graphene.14 The discovery of graphene nanosheets (GNS, 2010 Nobel Prize for Physics) in recent years has opened up an exciting field in science and technology of two-dimensional (2D) nanomaterials, with incessantly growing academic and technological innovations.23 GNS with a thickness of 1–10 nm, also known as graphene platelets (GPLs) or graphene nanoplatelets (GNPs), are comprised of a few layers of graphene and have similar features with monolayer graphene.20 The elastic modulus and inherent strength of GNSs are around 1 TPa and 130 GPa, respectively. In addition to the improved modulus of elasticity, GNSs can also improve the fracture toughness of any ceramic-based composite system by absorbing energy via the crack deflection and bridging.24–26 This suggests that GNSs can be an effective mechanical and biological reinforcement to HA. From a different perspective, the incorporation of various ions could improve the structural features, surface charge, and mechanical and biological properties of HA. Hence, special attention is focused to the anionic, cationic and multi-substituted HA for diverse biomedical applications.27–30 This article mainly focuses on the fabrication of HA–GNPs composites as ceramic-based bone graft substitutes and their mechanical behavior and in-vitro biofunctionality. We will first introduce previous studies on HA and related materials, followed by the fabrication aspects, physical properties, chemical properties, mechanical behavior, biofunctionality of HA–GNSs composites, and their applications. To conclude, a number of challenges and critical issues which need further research and elaboration are also discussed.

2. Literature review

2.1. Bioceramic materials

Different kinds of graft materials have been conventionally utilized for bone rehabilitations. In the late 1960s, due to their biomechanical properties, there was a great interest in developing ceramic materials as potential bone grafts and such biomaterials are also known as bioceramics. Bioceramics can be synthesized from various types of material, however, this article is restricted to CPs only, which have particular advantages due to the similarity to the mammalian endo-skeleton. There have been numerous important advancements in this field for the past decades. After the discovery of bioceramics compatible with the physiological environment, the current research is focused on the chemical formulations to establish direct chemical bonds with natural bones. Subsequently, by controlling the composition and structural features, it is possible to select the CPs-based implants which could be resorbed over time and biologically stable when incorporated into the endo-skeleton. These CPs-based scaffolds are porous and protect diverse biomolecules and cell tissues are currently designed to induce vascularization and bone formation. The CPs-based bio-ceramics used in biomedical applications consist of artificial bone grafts, bone augmentations, spinal fusion, maxillofacial reconstruction, bone fillers after tumor surgery, and periodontal disease repairs. Since they are promising carriers of growth factors, various types of
cells, and bioactive peptides, the future applications of CPs will most probably include drug delivery systems and modern tissue engineering purposes.31

As mentioned above, CaP salts are the main constituents of vertebrate endo-skeleton. The structure of a typical bone at various scales is shown in Figure 3, where the outer surface of bone is a dense matrix, while the interior consists of spongy and porous matrix with web-like trabeculae structure.

The compact natural bone is arranged into rings known as osteons, where the lymphatic vessels, nerves, and blood vessels are situated in the central Haversian canal. The lamellae rings encapsulate the Haversian canal. Cavities such as lacunae and canaliculi are actually micro-channels situated in the lamellae. The osteoblasts and osteocytes are situated in the exterior of the bone and in the lacunae, respectively. It is clear that the bone and other calcified tissues are classified as natural anisotropic composites, which are composed of biominerals embedded in water, organic materials, or in a protein matrix. The biomineral phase which is a type of CPs comprises of 65–70% of the bone, water consists of 5–8%, while the remaining portion consists of an organic phase in the form of collagen. The collagen, which endows the bone its elastic properties, is a matrix for the growth and deposition of minerals.32–34 HA is the thermodynamically most stable crystalline phase of CP, possessing the closest similarity to the natural bone.35,36 Consequently, synthetic HA has been considered as an artificial bone material for many years due to its good biocompatibility,37,38 biopolymer affinity,39,40 and osteogenic potential.41,42

2.1.1. Hydroxyapatite (HA) and related materials

The apatite group of minerals has the common formula M10(XO4)6Y2, where in HA, A = Ca, X = P, and Y = OH. HA is hexagonal (P63/m space group) with c = 0.6881 nm and a = 0.9432 nm, where the hydroxyl group is situated at the corners of the basal plane, at equidistant intervals perpendicular to the basal plane and along the columns parallel to the c axis. Figure 4 displays a c-axis projection of the atomic arrangement of Ca1, Ca2, PO4^3—, and OH— groups in the hexagonal crystal structure of HA and its modification by ionic substitutions. The hydroxyl groups are connected to six of the ten Ca2^+ ions, in the unit cell. All six of the phosphate (PO4)^3— tetrahedra are in a helical arrangement from z = 0.25 to z = 0.75. The PO4^3— tetrahedra forms the skeletal framework which accounts for the stability of the apatite structure.43,44

It has been well established that HA can initiate new bone generation via the osteoconduction mechanism without the presence of inflammation, systemic toxicity, or foreign body response. Upon the implantation of the HA-based material, a fibrous tissue comprising of carbonated apatite materializes on the surface and establishes the bonding between the implant and the natural bone. This leads to the fixation and stabilization of the implant in the tissue environment.45–48 Previous reports have confirmed that HA-based ceramics can be utilized to study the biomineralization in the human body.49–53 It has been demonstrated that HA particles could prevent the spread of various types of cancer cells.54,55 HA and related materials are commonly used in diverse biomedical applications, such as a replacement for periodontal and bone defects,56,57 ear implants,58 tissue engineering systems,59,60 alveolar ridge,61 dental materials,62 bioactive coating on osseous implants,63 and drug delivery agents.64 HA-based materials have also been used in many industrial, non-medical and technological applications such as catalysts for chemical reactions,65,66 host materials for lasers,67 gas sensors,68 ion conductors,69 and fluorescence materials.70 Besides, synthetic HA has been utilized in column chromatography,71,72 water treatment processes,73 and remediation of contaminated soils.74 Nanostructured HA (n-HA) mostly as HA nanoparticles, with proper morphology, stoichiometry,
and purity, have gathered huge interest in various biomedical applications. This configuration, which has a crystallite size smaller than 100 nm, exhibits ultrafine structure and high surface activity similar to the minerals in hard tissues. Accordingly, n-HA could promote osteointegration and subsequent bone regeneration. Nanosized HA-based ceramics have shown higher resorbability and bioactivity than micron-sized bioceramics. As reported in the literature, the release of Ca\(^{2+}\) from n-HA is similar to that of biological apatite but faster than any coarse crystals. Furthermore, n-HA possesses improved densification and sinterability owing to its high surface energy. This feature is able to prevent problems related to high-temperature sintering such as the formation of microcracks. It was found that n-HA is able to decrease apoptotic cell death, thus enhancing cellular activity related to bone growth and cell proliferation. The enhanced cellular differentiation and proliferation could be due to the superior surface properties of n-HA compared to the micron-sized HA. Hence, n-HA and its derivatives are considered as promising materials for diverse biomedical applications.

### 2.1.2. Substituted HA

As noted above, HA is widely utilized in biomedical applications due to its chemical similarity with the hard tissues. However, biological apatite, which is the main composition of the mineral phases in the mammalian endo-skeleton, differs from pure HA. Biological apatites are usually Ca deficient (Ca/P < 1.67) and are always carbonate substituted: \((\text{CO}_3)^{2-}\) for \((\text{PO}_4)^{3-}\). They are composed of small crystals with poor crystallinity and high solubility. Biological HA also has an important role in bone metabolism. Therefore, the preparation of partially substituted HA by these groups and elements to resemble the chemical composition of the natural bone has attracted much attention. As can be seen in Figure 4, substitutions for Ca\(^{2+}\), \((\text{PO}_4)^{3-}\), and \(\text{OH}^-\) groups in the HA structure are possible. These replacements alter the lattice parameter and the solubility. For instance, if \(\text{F}^-\) replaces the \(\text{OH}^-\) groups in HA, the
anions are closer to the Ca$^{2+}$ ions and contribute to the further stabilization of the structure, this suggests that fluoridation could decrease tooth decay.  

2.1.2.1. Cationic substitutions. To imitate the mineral constituent of natural bone, various types of substitutions, both cationic (substitution of calcium) and anionic (substitution of hydroxyl or phosphate groups), were reported with modifications in the chemical, physical, biological, and mechanical properties, from the in-vivo and in-vitro tests. In fact, the atomic doping or substitution in apatite is one of the strategies to overcome the intrinsic weaknesses of bioceramic implants. Many studies have reported that Ca$^{2+}$ sites in bioceramics can be replaced by various monovalent (K$^+$, Na$^+$, Ag$^+$), divalent (Cd$^{2+}$, Zn$^{2+}$, Eu$^{2+}$, Sr$^{2+}$, Mg$^{2+}$, Ga$^{2+}$ etc.), trivalent (Bi$^{3+}$, La$^{3+}$, Y$^{3+}$, Al$^{3+}$ etc.), tetravalent (Zr$^{4+}$), and pentavalent (Ta$^{5+}$, V$^{5+}$, Nb$^{5+}$) cations, which controls the implant-environment interactions. 

Replacements by bivalent ions in the apatite lattice do not cause charge imbalance. On the contrary, substitution by monovalent ions such as Na$^+$ and K$^+$ could lead to charge imbalance. But this type of charge imbalance could be neutralized by the creation of supplementary vacancies, or the simultaneous substitution of anions and cations without the loss of charge balance or formation of vacancies. The substitution of Ca$^{2+}$ ions by other cations could induce lattice alterations, in terms of expansion and contraction of the lattice parameters. It can be easily assumed that larger cations could increase the cell volume and expand the a-axis parameter. However, this is invalid, especially when a monovalent cation substitutes a bivalent cation. The channel diameter decreases with the formation of vacancies in the channel, and accordingly this decreases the a parameter. Site Ca2 is larger in volume than site Ca1 and it was reported that cations with a radius larger than Ca$^{2+}$ prefer to occupy site Ca2. Nevertheless, some studies have shown the contrary, in this regard the type of anion which occupies the channel becomes important. For example, in fluorapatite, the calcium ion (radius 0.099 nm) is substituted by the Mn$^{2+}$ ion (radius 0.080 nm) in site 1; however, the substitution occurs simultaneously at both sites in chlorapatite. The strength of the related bonds and the ionic charges control the overall distribution. The strength of the Metal–Oxygen interaction is another factor which controls the distribution of cations: site 1 allows the accommodation of larger cations due to the longer Metal (1)–O bond length. However, as the amount of the larger cation increases, the repulsion at the Metal (1) position extends of the c-axis, which is hindered by the metal atoms at site 2. The trivalent cations could be incorporated as lower-valenced hydroxo-ions to maintain the charge balance, where the ion-exchange ability depends more on the charge density than the ionic radii.

Among substituting cations, magnesium (Mg) as the fourth most common ion in the human body has been broadly examined. Actually, Mg$^{2+}$ ions are normally present at 0.44 wt% in natural enamel, 1.23 wt% in dentin, and 0.73 wt% in bone. Nonetheless, the quantity is around 2 wt% in the bone tissue and cartilage during the beginning of the osteogenesis process, but tends to decrease in the mature tissues. It has been shown that osteoporosis is directly related to magnesium deficiency, inhibition of skeletal metabolism stages, inhibition of osteoblastic and osteoclastic processes, bone growth, osteopenia, and increased bone brittleness. In terms of the crystallographic properties, Mg$^{2+}$ incorporation strongly distorts the HA lattice. Due to the smaller radius of Mg$^{2+}$ (0.069 nm) compared to Ca$^{2+}$ (0.099 nm), Mg incorporation into the apatite lattice decreases the c-axis dimension. Whereas according to Ren et al., both a and c reticular parameters decrease with the increase of Mg content. It has been reported that this substitution can only lead to a slight increase in the a lattice parameter. These inconsistencies are due to the strong influence of the preparation methods on the lattice parameters. With regards to the mechanical properties, Mg incorporation drastically decreases the microhardness and compressive strength up to a Mg content of 1.8 wt%, and leads to an increase in the fracture toughness up to a Mg content of 0.6 wt%. Furthermore, the substitution of Ca$^{2+}$ by Mg$^{2+}$ has a positive biological effect with enhanced biodegradability and solubility in physiological fluids, due to the lattice modifications.

On the other hand, strontium (Sr) is one of the most potent elements for bone ache and bone cancer relief, as well as osteoporosis treatments. This is due to the unique properties of Sr in stimulating osteoblast differentiation, inhibiting bone resorption and osteoclast activity. Therefore, strontium (strontium ranelate compound) has been broadly utilized for osteoporosis treatment, to enhance osteoblast proliferation, and to reduce the osteoclast differentiation. In vivo studies have also demonstrated that strontium ranelate inhibits bone resorption and promote bone formation. Sr incorporation also affects the HA mechanical properties, where the Sr-HA porous materials showed a compressive strength of 4.52 ± 1.40 MPa, similar to natural bone. Besides that, Kim et al. showed an increase in the Vickers value from 5.2–5.5 GPa for Sr-HA (Sr 8 mol%) compared to pure HA. From the biological assessments, the presence of Sr$^{2+}$ in the HA lattice has enhanced the bioactivity and
biocompatibility, subsequently promoting the osseointegration process.\textsuperscript{88,125–129}

Zinc (Zn) is also a vital element present in more than 300 types of enzymes. It is able to enhance DNA synthesis, enzyme activity,\textsuperscript{130} and exhibits antibacterial activity as with copper ions. With regards to the lattice parameters, the c-axis dimension of Zn-HA decreases with the increase of Zn content,\textsuperscript{131} due to the difference in the ionic radius of Ca\textsuperscript{2+} (0.099 nm) and Zn\textsuperscript{2+} (0.074 nm). In addition, Zn favors the β-TCP phase formation but inhibits the HA crystalization.\textsuperscript{132,133} It has also been reported that the thermal stability and the crystallinity of the apatite decline drastically with the increase of Zn content.\textsuperscript{131} Indeed, the presence of Zn increases the biological response,\textsuperscript{89} promotes osteoblastic proliferation, and prevents bone resorption by osteoclasts.

Silver (Ag) exhibits the strongest antibacterial strength at very low concentrations of 35 ppb without any toxic effects.\textsuperscript{134,135} In fact, Ag possesses high thermal stability, biocompatibility and non-toxicity towards living cells.\textsuperscript{136,137} The substitution of Ca\textsuperscript{2+} by Ag\textsuperscript{+} occurs preferentially at the Ca1 site of HA, results in an expansion of the lattice parameters,\textsuperscript{136,138,139} with the larger Ag\textsuperscript{+} radius (0.128 nm vs 0.099 nm). The Ag substitution in HA also increases the solubility and decreases the thermal stability.\textsuperscript{136,138,140} It is essential to determine the optimum amounts of Ag in the composite to achieve a perfect balance between cytotoxicity and effective antimicrobial activity.\textsuperscript{88} From the \textit{in-vitro} tests on human osteoblasts, Ag content between 2–4 wt\% provide a good balance between cytotoxicity and antimicrobial activity,\textsuperscript{141,142} whereas Ag content of 6 wt\% drastically slows the osteoblast growth, even causing osteoblast death.\textsuperscript{141}

Potassium (K) also influences the apatite nucleation and biomineralization processes.\textsuperscript{143,144} Besides that, sodium (Na) is also a trace element in the mammalian endo-skeleton, involved in the resorption, bone metabolism, and cell adhesion processes.\textsuperscript{145,146} The K\textsuperscript{+} ions are incorporated without large alterations to the lattice parameters.\textsuperscript{95,147,148} It would be expected that K\textsuperscript{+} substitution is favored at site Ca2 due to the larger K\textsuperscript{+} radius (0.133 nm), but the reverse was revealed:\textsuperscript{95} only 17\% of the K atoms are situated in site (II) while 83\% in site (I). Potassium incorporation enhances the thermal stability until 1300°C, and allows the thermal treatments at higher temperatures without HA degradation to porous and/or granulated materials.\textsuperscript{148} With regards to the Na-HA, it has been shown that the Ca2 is the most favored site for Na\textsuperscript{+} substitution.\textsuperscript{96} This modification stabilizes the conversion of β-TCP phase to the α-TCP phase until 1200°C.\textsuperscript{149} From the Rietveld refinements, it was shown that Ti incorporation expands both the α- and θ-axes. Wakamura et al.\textsuperscript{150} examined the bactericidal effects of Ti-HA and studied the photocatalytic decomposition of albumin and acetaldehyde under ultraviolet (UV) irradiation. In addition, Kandori et al.\textsuperscript{151} reported that thermally treated Ti-HA particles are potent photocatalysts for the decomposition of pathogenic proteins under UV irradiation. Manganese (Mn) also affects bone remodeling while Mn deficiency delays the endochondral osteogenesis which decreases the organic matrix synthesis.\textsuperscript{152} Integrins are a group of receptors which mediate cellular interactions with surface ligands and the extracellular matrix is activated by Mn\textsuperscript{2+}. Therefore, Mn\textsuperscript{2+} increases the ligand affinity towards integrin, consequently encouraging the cell adhesion.\textsuperscript{153,154} The Mn-substituted HA showed no cytotoxic effects with osteoblasts in the \textit{in-vitro} tests.\textsuperscript{155} Iron (Fe) compounds absorb phosphate ions and salivary calcium, while promoting apatite nucleation, hence favors the replacement of minerals dissolved during the caries process. Some iron compounds with magnetic properties are also utilized in diverse biomedical applications. Wu et al.\textsuperscript{156} and Li et al.\textsuperscript{155} studied the biocompatibility of Fe\textsuperscript{3+} substituted HA powders via the \textit{in-vitro} tests with osteoblasts. Fe-HA is also utilized as a heating mediator in hyperthermia therapy of cancer.\textsuperscript{157} The \textit{in-vivo} tests of mixed Fe-HA particles around a tumor gave a marked decline in the tumor volume in PBS.\textsuperscript{158} Lanthanides have been widely used as luminescent probes in \textit{in-vivo} imaging and magnetic resonance imaging due to their long emission lifetimes and narrow emission bands.\textsuperscript{159,160} In addition, gallium (Ga) has interesting biological features and is utilized in the treatment of certain disorders.\textsuperscript{161} From the literature, Ga\textsuperscript{3+} does not substitute Ca\textsuperscript{2+} owing to the heterovalent substitution, hence does not distort the lattice. Although the biological responses of synthetic Ga-HA have not been widely examined, Ga-HA could be utilized to stimulate bone growth.\textsuperscript{88} Copper (Cu), is present in several metabolic pathways, an important micronutrient in living organisms, with antibacterial properties used in the sterilization of chest wounds and drinking water.\textsuperscript{162} The antibacterial property of Cu-HA against \textit{C. albicans} fungi and \textit{E. coli} bacteria has been reported, which shows a strong antimycotic activity.\textsuperscript{163} In addition to the above-mentioned dopants, cobalt is also an essential element as a constituent of vitamin B12, required for the myelin sheath formation of neurons and neurotransmitters, DNA synthesis in cells, and red blood cell production.\textsuperscript{164} So, Co\textsuperscript{2+} ions can also be utilized as antiviral and antibacterial agents in a variety of organic complexes.\textsuperscript{165,166}
2.1.2.2. Anionic substitutions. As shown in Figure 4c, four different crystallographic orientations are present in the apatite unit cell. The Ca1 position consists of four calcium ions, which are surrounded by nine oxygen atoms in columns parallel to the c-axis. The Ca2 position consists of six calcium ions per unit cell, which forms two triangles along the c-axis, at \( z = 1/4 \) and \( 3/4 \). These triangles accommodate two monovalent anions (\( \text{Cl}^-, \text{OH}^-, \text{F}^- \)) and also a bivalent \( \text{CO}_3^{2-} \) per unit cell, are also known as the anion channel. The larger size of the Ca2 triangle enables the anion movement along the column axis. The six \( \text{PO}_4^{3-} \) anions form the tetrahedra, where the four oxygen atoms are coordinated to the central P atom.\(^{30}\) Although the \( \text{OH}^- \) in the anion channel can be substituted by the monovalent anions without any charge imbalance, the bivalent anions (e.g., \( \text{HPO}_4^{2-}, \text{CO}_3^{2-}, \text{SO}_4^{2-}, \text{SeO}_3^{2-}, \text{SeO}_4^{2-} \)) can also substitute the phosphate, by balancing the charge of the calcium and hydroxide groups. The negative charges in the HA are compensated by the hydroxide groups when substituted by tetravalent anions (e.g., \( \text{SiO}_4^{4-} \)).\(^{88}\)

The major substituent in biological apatite is carbonate \( (\text{CO}_3^{2-}) \), a constituent of bone mineral, typically 4–8 wt%.\(^{100,167–169}\) Even though carbonates are related to the disordered regions in the apatites, their exact locations are unclear.\(^{170}\) Nevertheless, it is generally accepted that carbonates substitute the phosphates in biological apatites.\(^{171}\) In general, the \( \text{CO}_3^{2-} \) group can be accommodated at two sites in the lattice, i.e., the \( \text{OH}^- \) position as A-type and the \( \text{PO}_4^{3-} \) position as B-type carbonated apatites.\(^{172}\) The B-type substitution is most preferred when the A/B ratio is between 0.7–0.9.\(^{173}\) The A/B ratio in the natural bone is dependent on the maturation.\(^{174}\) This type of anionic substitution is responsible of the following characteristics: a decrease in the a-axis length along with a rise in the c-axis length, alterations in crystallite size and microstrains, mechanical reinforcement of the bone and optical birefringence,\(^{175}\) and increased solubility\(^{176}\) due to the weaker Ca–CO\(_3\) bonds compared to the Ca–PO\(_4\) bonds. From a biological standpoint, the \( \text{CO}_3^{2-} \) ions play an important role in bone metabolism, thus is the main reason for the utilization as bone graft materials. It has been shown that this type of substitution could enhance the material bioactivity.\(^{177–179}\) Besides that, \( \text{CO}_3^{2-} \) substitution can improve the bone apposition rates. Spence et al. showed that carbonated HA increased the collagen generation by human osteoblast cells, compared to the undoped HA.\(^{180}\)

On the other hand, fluoride (\( \text{F}^- \)) is necessary for normal dental and skeletal growth.\(^{181}\) The \( \text{F}^- \) ions could promote in-vitro extracellular matrix formation and increase in-vivo bone union, enhancing the osteoblastic activities in cell differentiation and proliferation.\(^{182}\) The \( \text{F}^- \) incorporation in the anion channels occurs readily,\(^{183}\) at \( z = 1/4 \) and \( 3/4 \), causing a shrinkage along the a-axis of the unit cell.\(^{184}\) This effect is due to the smaller radius of \( \text{F}^- \) \((0.119 \text{ nm})\) compared to the \( \text{OH}^- \) \((0.153 \text{ nm})\).\(^{185–188}\) However, the fluoride substitution expands the c-axis parameter as a result of the larger crystal size.\(^{187}\) In bone graft applications, F-HA is an alternative material for bone rehabilitation, from its good biocompatibility, lower solubility, better chemical, and thermal stability.\(^{189}\)

Since the replacement of \( \text{OH}^- \) by \( \text{Cl}^- \) ions increases the acidity and leads to the solubilization of alkaline salts, pure chlorapatite is not a good biomaterial. The chloride (\( \text{Cl}^- \)) content in natural bone could reach 0.13 wt%, while is 6.8 wt% in pure chlorapatite, therefore chloride-substituted HA (CIHA) could be suitable for biomedical applications.\(^{30}\) Due to the formation of an acidic environment, the presence of \( \text{Cl}^- \) is very important in the organic matrix digestion and bone resorption process.\(^{184}\) This suggests that CI-HA is highly considered in bone tissue applications due to the improved bioactivity and osteoconductivity compared to pure HA.\(^{190}\)

Inorganic sulfates play a key role in cell matrix synthesis, cell growth, and cell membrane maintenance,\(^{191}\) and have been utilized in the treatment of hypercalcemia.\(^{192}\) It has been found that the incorporation of \( \text{SO}_4^{2-} \) inapatite decreased the crystallinity but increased the structural disorder.\(^{193}\)

The physiological presence of selenium (Se) is important for the proper functioning of the body. It provides protection against carcinogenesis and oxidative stress, and possesses antibacterial properties.\(^{194,195}\) It is a constituent of seleno-proteins and glutathione peroxidase enzyme, needed for the cell membrane protection against harmful factors.\(^{196}\) It has been reported that selenium plays a key role in the induction of cancer cell apoptosis and in protein functions.\(^{197}\) The antibacterial property of Se is mainly due to the oxidative stress, damaging the bacterial cell walls.\(^{195,198–200}\) For this reason, Se compounds have been used for anticarcinogenic functions and as multifunctional biomaterials.\(^{201}\) The lattice parameters are only slightly influenced by selenate ion substitution, due to the \( \text{SeO}_4^{2-} \) ion having the same structure as the \( \text{PO}_4^{3-} \) ion (i.e., tetrahedral), but with a slightly larger diameter \((0.249 \text{ nm vs. } 0.238 \text{ nm})\).\(^{202}\) Due to the strong anticarcinogenic properties of selenium,\(^{197,203}\) Se-HA can be employed as scaffolds for new bone tissue growth, the inhibition of tumor cell proliferation and in bone cancer treatments.

Silicon is an element essential to the metabolic processes of connective tissues and bones, which play crucial roles in the osteoblast proliferation and differentiation, the biomineralization and remodeling processes, the osteoblast collagen synthesis, as well as the osteoclast
development and resorption activities.\textsuperscript{204,205} With regards to the lattice parameters, an increase in the \(a\) and \(c\) reticular constants is observed, from the larger ionic radius of \(\text{Si}^{4+} (0.42 \text{ Å})\) compared to the \(\text{P}^{5+} (0.35 \text{ Å})\).\textsuperscript{82} Although the \(\text{Si}^{4+}\) ionic radius is larger than the \(\text{P}^{5+}\), the \(\text{P} = \text{O}\) and \(\text{P}-\text{O}\) bonds (0.155 nm) are shorter than the \(\text{Si}-\text{O}\) bonds (0.161 nm), thus the \(\text{SiO}_4\) tetrahedra is actually larger than the \(\text{PO}_4^{3-}\) tetrahedra.\textsuperscript{206} In many previous studies, an improved bioactivity of \(\text{Si}\)-doped HA has been reported,\textsuperscript{178,207–211} emphasizing that \(\text{Si}\) could induce \textit{in-vitro} osteoblast cell activity.\textsuperscript{211} Balamurugan et al.\textsuperscript{214} examined the \textit{in-vitro}\ osteoblast on Si-HA with different \(\text{Si}\) contents (i.e., 1, 3, and 5 mol\%), where 3 mol\% \(\text{Si}\) was the optimal value.

### 2.1.2.3. Multi-ionic substitutions

In addition to cationic and anionic substitutions, numerous works have reported multi-ionic substitutions that mimic the structural features and chemical composition of bioapatites. The multi-ionic substitutions in HA provide new physical, chemical, biological, and mechanical properties compared to pure HA or mono-ionic substituted HA ceramics.\textsuperscript{27–30} Kannan et al.\textsuperscript{212} prepared magnesium and sodium co-substituted hydroxyapatite (Na/Mg-HA) and \(\beta\)-TCP mixtures via the aqueous precipitation method. The lattice parameters had a more contracted \(a\)-axis and expanded \(c\)-axis. Kalita et al.\textsuperscript{213} synthesized HA substituted Zn and Mg via the sol-gel method, and showed that the presence of substituents improved the surface hardness and compressive strength. Besides that, biodegradation tests conducted in SBF indicated that the presence of dopants in n-HA decreased the resorption rate and surface hardness degradation. Gopi et al.\textsuperscript{214} reported the successful preparation of Zn, Mg, and Sr multi-substituted hydroxyapatite (Sr/Mg/Zn-HA) and found that these substitutions promoted apatite growth and accelerated growth on to itself. Gopi et al.\textsuperscript{215,216} produced strontium/cerium co-substituted HA (Sr/Ce-HA) and also synthesized bioactive and antibacterial silver/magnesium co-substituted HA (Ag/Mg-HA) with a range of Ag concentrations (0.5, 1.5, 2.5 wt.%). The \textit{in-vitro} antibacterial activity tests indicated that Mg decreases the cytotoxicity of Ag.\textsuperscript{216} In another work, Hu et al.\textsuperscript{217} synthesized Ti\(^{4+}\) co-substituted hydroxyapatite (Ti/Ag-HA, Ti/Cu-HA, Ti/Zn-HA) by the co-precipitation and ion-exchange methods. The doping of antibacterial ions (Ag\(^{+}\), Cu\(^{2+}\)) in Ti/Ag-HA and Ti/Cu-HA gave highly efficient elimination of E. coli in the dark and S. aureus in weak UVA irradiation. Furthermore, several studies have dealt with the anionic co-substitution and anionic-cationic co-substitutions in the apatite lattice. For instance, Kannan et al.\textsuperscript{186} synthesized Cl/FHA via aqueous precipitation with chlorine and fluoride concentration between 0.79–3.21 wt.% and 0.41–1.79 wt.\% respectively. Also, given that carbonates are the most common substituents in bioapatite, the co-substitutions of Mg\(^{2+}\) Na\(^{+}\), and CO\(_3\)^{2–} ions are a common practice\textsuperscript{218–220} in native bone bioapatite. However, in most cases, these results are still far from commercial applications and much work remains to be done from fundamental studies to clinical execution.

### 2.2. Carbon nanostructures (CNSs)

Much of nanotechnology advancements are centered on carbon nanostructures. The reputation of CNSs is built on fullerenes and carbon nanotubes due to their early discoveries, nevertheless other members of the nanocarbon family are also attracting attention.\textsuperscript{221} In this regard, the structure, properties, and several applications of nanostructured graphite, has been presented in a book by Inagaki.\textsuperscript{222} New carbon allotropes such as carbolite, a chain-like crystalline form of carbon, are being discovered. Undoubtedly, carbon nanoscience, a discipline encompassing all carbon polymorphs at the nanoscale is becoming a new research field by itself.\textsuperscript{221} There is now a strong tendency to unite researchers from different carbon disciplines such as graphite fibers, fullerenes, and diamond, which have been developing independently.\textsuperscript{223} Although there are excellent reviews\textsuperscript{224,225} that discuss discoveries, nevertheless other members of the nanocarbon family are also attracting attention.\textsuperscript{221} In this regard, the structure, properties, and several applications of nanostructured graphite, has been presented in a book by Inagaki.\textsuperscript{222} New carbon allotropes such as carbolite, a chain-like crystalline form of carbon, are being discovered. Undoubtedly, carbon nanoscience, a discipline encompassing all carbon polymorphs at the nanoscale is becoming a new research field by itself.\textsuperscript{221} There is now a strong tendency to unite researchers from different carbon disciplines such as graphite fibers, fullerenes, and diamond, which have been developing independently.\textsuperscript{223} Although there are excellent reviews\textsuperscript{224,225} that discuss several classes of CNSs, the graphene family especially rGO and GNPs has remained out of the scope of most recent discussions. Therefore, this topic is reviewed in this section.

In general, the element carbon is able to form multiple allotropes and exhibits different properties due to its versatile bonding and atomic arrangements, as shown in Figure 5. The discovery of fullerene in 1985 spurred an exciting new research on carbon allotrope such as carbon nanotubes and graphene.\textsuperscript{226,227} Before these discoveries, diamond (three-dimensional \(sp^3\)-hybridized carbon atoms), and graphite (two-dimensional \(sp^2\)-hybridized carbon atoms) are the well-established forms of carbon.\textsuperscript{228} More carbon allotropes such as nanotubes, fullerenes, and graphene (\(sp^2\)-hybridized single layer carbon atoms), lonsdaleite, and \(C_8\) (\(sp^2\)-hybridized carbon atoms) have been discovered; each have unusual properties different from the traditional forms of carbon such as diamond and graphite.\textsuperscript{226,229–232} The \(sp\) hybridization of carbyne brings a one-dimensional form of carbon into existence.\textsuperscript{226} Many CNSs have recently gathered interest in fields of biology and medicine. Indeed, due to their particular properties, fullerenes, and CNTs have been used for several therapeutic and pharmaceutical purposes since the mid-1990s,\textsuperscript{228} while other CNSs such as...
Graphene, GO and rGO have become popular in the past decade.\textsuperscript{14,221,233,234}

### 2.2.1. Graphene

Graphene is a single layer of sp\textsuperscript{2} hybridized carbon atoms in a 2D honeycomb lattice, is a building block for other forms of carbons such as graphite, fullerenes, and carbon nanotubes.\textsuperscript{235} Graphene was thought as non-existent in a free state until 2004, when a single-atom-thick layer of carbon was isolated.\textsuperscript{236} This led to the incessant interest in graphene, due to the amazing physical and chemical properties.\textsuperscript{237} Recently, many research efforts were spurred by graphene, and supported by large funding from China and the European Union.\textsuperscript{235} In spite of the wide range of applications of graphene compared to other forms of carbon, it is still unclear whether graphene could be the next revolutionary material (Table 2).\textsuperscript{235} Graphene has exclusive optical and electronic properties,\textsuperscript{233} due to the continuous and unbroken sp\textsuperscript{2}-bonded carbon atoms,\textsuperscript{238} which also endows it with excellent mechanical properties. The elastic modulus and intrinsic strength of graphene are estimated between 60–130 GPa and 1 TPa, respectively.\textsuperscript{239} Graphene is a flat sheet of carbon atoms of a single atom thickness,\textsuperscript{240} thus provides more contact area with the host material, as both the top and bottom surfaces of a single graphene sheet are able to interact with the host material. This is a clear advantage compared to other folded nanocarbons such as fullerenes and CNTs. The amount of surface area and aspect ratio (ratio of the lateral size and thickness)

![Figure 5. Schematic images of the three kinds of hybridization of carbon to form allotropes. (© American Association for the Advancement of Science. Reproduced with permission from B. Pan et al.\textsuperscript{226} Permission to reuse must be obtained from the rightsholder.) (© Elsevier. Reproduced with permission from S.F. Kiew.\textsuperscript{227} Permission to reuse must be obtained from the rightsholder.)](image)

#### Table 2. Graphene features with respect to other carbonaceous materials. (© Nature Publishing Group. Reprinted with permission from Raccichini et al.\textsuperscript{235} Permission to reuse must be obtained from the rightsholder.)

<table>
<thead>
<tr>
<th></th>
<th>Graphene</th>
<th>Carbone nanotube</th>
<th>Fullerene</th>
<th>Graphite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimensions</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Hybridization</td>
<td>sp\textsuperscript{2}</td>
<td>Mostly sp\textsuperscript{2}</td>
<td>Mostly Sp\textsuperscript{2}</td>
<td>sp\textsuperscript{2}</td>
</tr>
<tr>
<td>Hardness</td>
<td>Highest (for single layer)</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Tenacity</td>
<td>Flexible, elastic</td>
<td>Flexible, elastic</td>
<td>Elastic</td>
<td>Flexible, non-elastic</td>
</tr>
<tr>
<td>Experimental SSA (m\textsuperscript{2} g\textsuperscript{-1})</td>
<td>(\sim 1500)</td>
<td>(\sim 1300)</td>
<td>80–90</td>
<td>(\sim 10–20)</td>
</tr>
<tr>
<td>Experimental conductivity (S cm\textsuperscript{-1})</td>
<td>(\sim 2000)</td>
<td>Structure-dependent</td>
<td>10\textsuperscript{-10}</td>
<td>Anisotropic: 2–3 × 10\textsuperscript{5}\textsuperscript{6}</td>
</tr>
<tr>
<td>Thermal conductivity (W m\textsuperscript{-1}K\textsuperscript{-1})</td>
<td>4840–5300</td>
<td>3500</td>
<td>0.4</td>
<td>Anisotropic: 1500–2000\textsuperscript{6}, 5–10\textsuperscript{7}</td>
</tr>
</tbody>
</table>

\(^{a}\) a direction,

\(^{c}\) c direction.
of a single layer of graphene can reach 2600 m² g⁻¹, which is much higher than those of CNTs and fullerenes. These features can generate more active sites for expediting chemical and physical interactions, subsequently improving the bonding between graphene and the host material. On the other hand, in view of the fact that carbon is one of the most abundant elements in the earth, CNSs are biologically and environmentally friendly than most organic and inorganic materials. Graphite, in particular, is a naturally occurring compound which has been utilized for hundreds of years without any toxic side effects. Therefore, it is expected that graphene would be also secure and effective for biological applications.

Graphene is currently extending its utilization in biomedical topics such as graphene-enhanced cell growth and differentiation, precise biosensing through graphene-quenched fluorescence and graphene-assisted laser desorption/ionization in mass spectrometry. Accordingly, studies on the use of graphene materials in orthopedic applications have grown exponentially in recent years.

2.2.2. Graphene oxide (GO)

GO is a type of functionalized graphene, a single layer of sp²-hybridized carbon atoms but with the presence of carboxyl, carbonyl, hydroxyl, and epoxy functional groups, attached to the edges and the basal planes of the graphene sheets. The presence of the functional groups changes the van der Waals interactions between the sheets, thus improving the dispersion in water, N-methylpyrrolidone, dimethylformamide, tetrahydrofuran and other polar solvents (Figure 6). In general, GO has large surface area, high aspect ratio and tensile strength. With the enhanced mechanical properties and the large dispersion in water, GO has become a promising material for improving the mechanical properties of composites. Although GO has been broadly studied in polymeric composites, the orthopedic applications of GO is still new.

2.2.3. Reduced graphene oxide (rGO)

rGO is also another type of functionalized graphene, and is usually prepared from GO by using chemical reductants such as hydrazine, dimethyl hydrazine, hydroquinone, sodium borohydride, hydroiodic acid, sulfur-containing compounds, ascorbic acid, and vitamin C. Among them, hydrazine is extensively utilized because it is an effective reducing agent and well suited for the reduction of GO in various media. However, the hydrazine reduction process is slow, toxic and unstable. Accordingly, efforts have been made to employ other reducing agents, such as hydroquinone and sodium borohydride. However, these chemicals are also toxic and slightly explosive. Therefore, green methods utilizing natural products for the reduction of GO are seriously taken into consideration. Fernandez-Merino et al. reported the reduction of GO to rGO utilizing vitamin C as the reductant while Zhu et al. utilized reducing sugars for the same purpose. In another work, Wang et al. reported the reduction of GO by using tea polyphenol as the reductant. It should be noted that the stability and solubility of the rGO suspension is also a problem, as rGO easily precipitates from suspensions. Hence, a low-cost, effective, and green reductant for
the chemical synthesis of rGO in bulk quantities is still very much in consideration.\textsuperscript{267}

\subsection*{2.2.4. Graphene nanoplatelets (GNPs)}

As shown in Figure 6, GNP are multi-layered graphene consisting of 10–30 sheets of graphene. This type of CNSSs is composed of short stacks of platelet-shaped graphene sheets that are similar to CNTs, but in planar form.\textsuperscript{268,269} The chemical functionalization of the edges of the platelets could enhance the hydrogen or covalent bonding ability. Improved mechanical properties and enhanced barrier properties (e.g., surface hardness, stiffness, and strength) can be attained with GNPs due to their morphology and size.\textsuperscript{268,270} This configuration also promotes excellent thermal and electrical conduction due to its pure graphitic composition. Naturally, GNPs carry functional groups such as ethers, carboxyls, or hydroxyls which react with humidity to form acids or other compounds. These functional groups are situated at the edges of the particles and their weight percent alters with the particle size. Due to their unique nanoscale features (shape and size) and material composition, GNPs can be utilized to improve the properties of a wide range of advanced materials. These multifunctional enhancements by GNPs make them ideal precursors for advance material applications.\textsuperscript{268}

\section*{2.3. Synthesis of HA, CNSs, and their composites}

\subsection*{2.3.1. Synthesis of nanosized HA}

The synthesis of HA particles is the first step to fabricate HA-based bone grafts. Depending on the requirements (e.g., crystallinity, particle size, specific surface area, and morphology), different methods can be employed to synthesize HA. Methods such as solid-state reaction,\textsuperscript{271} chemical precipitation,\textsuperscript{272} sol-gel process,\textsuperscript{273} hydrothermal method,\textsuperscript{274} emulsion route,\textsuperscript{275} sonochemical,\textsuperscript{276} solution combustion,\textsuperscript{277} biogenic sources,\textsuperscript{278} flame spray pyrolysis,\textsuperscript{279} and microwave-assisted synthesis\textsuperscript{280} have been utilized to prepare HA particles (Figure 7).\textsuperscript{32}

There are three methods of HA synthesis: low temperature (aqueous precipitation), high temperature (hydrothermal), and high energy (ultrasonic irradiation) explained in the following paragraphs. The wet chemical precipitation methods are one of the most widespread approaches for the preparation of HA nanoparticles due to their availability, simplicity, the use of inexpensive precursors,\textsuperscript{32} lower reaction temperatures, and low operating costs. In addition, this process is suitable for manufacturing applications due to the scalability. The precipitation method involves the mixing of reactants in water at controlled atmosphere, temperature, pH, and allowing the precipitate to age under continuous stirring. Once aged, the precipitate is thoroughly washed, filtered, and dried (Figure 7b). The crystal growth and nucleation occur upon supersaturation; this occurs when the calcium solution is titrated by the phosphate solution forming precipitated particles. To avoid the formation of secondary phases during the aqueous precipitation, it is essential to carefully control the experimental parameters such as the concentration of reactants, reaction temperature, atmosphere, and pH. In this approach, the pH plays an important role in controlling the properties of the HA nanoparticles. The HA is insoluble at pH larger than 8. When the pH is larger than 8, the nucleation rate of the HA particles increases with the increase of pH. When the nucleation rate increases, the crystals nucleate in a short period of time and the growth of the HA crystals is restricted, thus favorable for the generation of HA nanoparticles. The pH value can be controlled between 9 and 11 by the addition of aqueous ammonia.\textsuperscript{32,281–283} The hydrothermal method has been used to synthesize materials at high pressure and high temperature conditions in super-saturated solutions.\textsuperscript{284} The hydrothermal reaction involves the use of water as the solvent (with precursor soluble ions) heated in a sealed vessel. The temperature of the mixture can exceed the boiling point of water as the pressure in the vessel exceeds the ambient pressure. The experimental variables can be controlled to a larger degree by changing the solvent and reactant properties (e.g., solubility) at elevated temperatures. The reaction is more predictable when the crystal growth, nucleation, and aging can be regulated (Figure 7d). It should be noted that calcination is not required in this method. The low temperature methods such as sol-gel synthesis and wet chemical precipitation require post heat treatment for the crystallization of HA; but HA crystallization can be performed in a single step via the solvothermal and hydrothermal methods. The main advantages of these methods are the use relatively low-cost reagents, short reaction times, and yields of nearly 100%. Furthermore, micro and nanotube HA, as well as HA nanorods with micron lengths have been prepared by this process.\textsuperscript{32,285–287}

Sonochemical methods which are based on the chemical reactions perpetuated by powerful ultrasonic irradiation always yield nanosized products. Figure 7f shows the main mechanism behind the sonochemical method, which is the acoustic cavitation where the formation, growth, and collapse of micro-bubbles occur.\textsuperscript{32} The chemical reactivity is accelerated by the heterogeneous reactions in the liquid and solid phases. Recently, it has been revealed that sonication raises the rate of HA crystallization up to 5.5 times.\textsuperscript{288} The HA nanoparticles synthesized by the sonochemical method are smaller, more uniform, and have crystals with lower agglomeration. All of these characteristics are dramatically influenced by
various parameters such as the irradiation time, concentration of additive, and subsequent thermal treatment.\textsuperscript{32,289} Briefly, nanoparticles synthesized by the sonochemical method can significantly enhance the sintering kinetics and the mechanical properties of the final product, due to the higher surface area.

### 2.3.2. Synthesis of CNSs

The most common methods adopted for graphene production are shown in Figure 8.\textsuperscript{235} Due to the high production costs and limited scalability, methods such as mechanical exfoliation,\textsuperscript{236,290} the utilization of SiC substrate,\textsuperscript{290,291} and organic precursors\textsuperscript{290,292} inevitably restrict graphene to fundamental and niche applications only, such as high-frequency transistors and touch screens.

The chemical vapor deposition (CVD) of hydrocarbons,\textsuperscript{291} is a well-established technique in industry, but is inappropriate for the mass-production of graphene due to the moderate purity, lower yield and the high cost.\textsuperscript{290} However, CVD is an effective method to produce
vertically oriented graphene nanosheets, even though the packing density is very low. Apart from the CVD technique, two other methods that are widely utilized for the bulk production of graphene are the reduction and liquid-phase exfoliation of GO. The pristine or expanded graphite are obtained by the thermal expansion of graphite intercalated compounds by the liquid-phase exfoliation method, followed by the dispersion in a solvent to weaken the van der Waals attraction. Subsequently, an external perturbation such as ultrasonication, electric field, or shearing is utilized to exfoliate graphite to graphene sheets. But the lower yield of this process is due to the presence of substantial amount of unexfoliated graphite. Nonetheless, the lower cost of liquid-phase exfoliation and higher scalability are suitable for the bulk production of graphene. In the second approach, GO with an interrupted sp²-bonding network, is obtained by the strong oxidation of pristine graphite followed by ultrasonication and stirring in liquid media. GO must be reduced to rGO to restore the π network, which is the main characteristic of conductive graphene. Electrochemical, thermal, and chemical processes are employed to prepare rGO from GO. These methods produce graphene in bulk quantities with higher yield and lower costs, despite the lower quality of the product with intrinsic defects (deformations and edges) and extrinsic defects (H- and O-containing groups). Although these are the methods for producing commercial graphene, other approaches are also available such as direct arc-discharge carbon nanotube unzipping. However, on account of their higher costs, these methods remain marginal, thus inappropriate for bulk production.

The ultrasonic exfoliation of various forms of graphite usually gives GNPs with a wide distribution of thicknesses. The concentration of the GNPs in dispersions obtained by this method is very small, between 0.01–0.1 g/l. In addition, the content of surfactant in the dispersions is usually much higher than the content of graphene itself, for this reason the mass production of graphene by this method is rather expensive. At the first sight, the intercalation of foreign particles between the layers of graphite could weaken the bond between them and allow an easier exfoliation. This might work with covalent attachment of fluorine atoms to the graphene skeleton. However, almost all known intercalants capable of penetrating between the graphite layers are strong electron donors or acceptors and form charge transfer or ionic structures with the graphene layers, i.e., the increased bonding strength between the graphene layers due to electrostatic forces between the layers. However, many graphite intercalation compounds are able to undergo secondary thermal or chemical transformations that result in a profound restructuring of the crystal structure, accompanied by the exfoliation of crystalline intercalated compounds to GNPs and even graphene monolayers. The most common example is the thermal expansion of graphite intercalation compounds (mostly graphite sulfate or nitrate), which leads to the formation of expanded graphite. Thermally expanded graphite may be dispersed by sonication to GNPs in alcohol solution. However, the GNPs obtained by this way are relatively thick, in the order of 50 nm. Highly exfoliated thermally expanded graphite (HETEG) can be split into much finer nanoplatelets. The HETEG is easily dispersed in organic solvents or in aqueous solutions of sodium dodecylbenzenesulfonate (C₁₈H₂₉NaO₃S), and forms one-, two-, and few-layered graphene. However, this method has a serious disadvantage, the use of very toxic and dangerous reagents and by-products, so it is hardly acceptable for the bulk preparation of graphene. It has also been reported that microwave radiation of alcoholic suspensions resulted in GNPs with thickness of about 10 nm. In some cases, the preparation of GNPs via an expanded graphite peroxosulfate compound has been reported, where the intercalation of graphite with a solution of ammonium persulfate in...
anhydrous sulfuric acid gives finer GNPs compared to the intercalation in concentrated (95%) sulfuric acid.306

2.3.3. Synthesis of HA-CNSs composites
In most operating conditions, single-phase ceramics with lower mechanical strength or lower bioactivity cannot satisfy multiple requirements. The development of composite materials which encompass the properties of every phase is an excellent approach to improve the quality of bone rehabilitation materials. Due to its excellent biocompatibility and bioactivity, HA has been used as a drug releasing agent and a rehabilitation material for human hard tissues. However, the lower strength and inherent brittleness of HA places obstacles to load-supporting applications. Different types of approach, such as incorporation of reinforced phases and microstructural refinement of HA particles, have been employed to improve the fracture toughness and flexural strength of HA ceramics. These types of HA composite are as follows.

2. HA-polymer composites (HA-PLLA, HA-PGA, HA-PPF).313–316
4. HA doped with metal ions.30,320

With regards to the HA-Al2O3 composite, although the fracture toughness and flexural strength of the composite are improved significantly, the flexural strength stability is still poor. This is due to the wide differences in the thermal expansion coefficients of HA and Al2O3, which could lead to multiple micro-cracking after the sintering process, thus weakening the flexural strength of the composites.321 Due to the unique mechanical properties of CNSs, they should form excellent composites with HA. However, the widespread use of CNSs as fillers may be decreased by factors such as inhomogeneous dispersion in the matrix, which can influence the mechanical properties and cytotoxic response in an organic environment. Stoichiometric HA had shown limited reactivity both in in-vitro322 and in-vivo323 experiments, which causes longer recovery times.324 The lower osteogenic capacity and mechanical properties of synthetic HA is attributed to the absence of trace elements in the natural bone.325 Therefore, several attempts have been made to incorporate these elements into synthetic HA to improve the mechanical and biological properties.

Doping is the introduction of elements (molecules, atoms, or ions) to a targeted material to improve the properties. The term impurity is used for naturally occurring foreign elements in a material and not due to a controlled procedure. There are numerous reports of accidental impurities becoming good dopants, enhancing the properties or introducing new properties to a material. A doping element or dopant is found at very low concentrations, between few ppm to few percent.326 In synthetic HA, trace elements such as anions and cations play important roles in enhancing the biological and mechanical performances of the bone.138,320,327–329 The doping of cations leads to changes in the structure, microstructure and surface properties of HA. It has been found that the increase in the valency of dopants generally results in the increase of bioactivity of the doped HA. A desired level of biocompatibility, bioactivity, adsorption, and solubility properties can be achieved by controlling the substitution of Ca2+, PO43−, and OH− in the HA lattice. Numerous studies have reported the synthesis of substituted apatites using the wet chemical precipitation,108,330,331 sol-gel,213,329,332 and hydrothermal333 methods with different chemical precursors. For instance, Nedelec et al.326 mentioned the advantages of the sol–gel process in the preparation and doping of ions. Homogeneous doping often yields products with homogeneous properties and is fundamental for the large-scale production with low doping levels. Another advantage of the sol–gel method is that the variation of the concentration and nature of the doping ion can be accomplished easily.

In general, one of the major challenges in composite system is the homogeneous distribution and efficient use of the secondary phase. The high surface energy of GNPs is responsible for the agglomeration when added as the reinforcing phase in composites, which causes a negative influence on the reinforcing ability.269 Accordingly, several modifications have been proposed to optimize the distribution of GNPs in HA. The main goals are: (i) to avoid agglomeration but enhance dispersion of GNPs in the HA matrix; and (ii) better interfacial interaction between the GNPs and HA for stronger bonding in the composite.

The chemical precipitation of HA on GNPs surface can be accomplished by the dispersion of the GNPs in a chemical bath where the HA is precipitated. Numerous studies report the dispersion of GNPs in calcium nitrate/chloride/hydroxide/carbonate bath, followed by stirring. This is followed by the drop-wise addition of diamonium hydrogen phosphate/phosphoric acid to the bath to precipitate the HA on the suspended GNPs. The control of the precipitation parameters, i.e., temperature and pH > 10, ensures the precipitation of the HA phase and prevents the formation of the CaHPO4·2H2O and Ca3(PO4)2 phases. Previous studies have reported the uniform distribution of GNPs in HA via the chemical precipitation method.334–338 The chemical precipitation of HA on GNPs surface is reported to have an amorphous structure that requires post treatment to the
crystalline phase. Therefore, some studies reported the formation of highly crystalline HA/graphene by the hydrothermal method. To prevent the agglomeration of GNPs, some reports suggest the use of DMF and CTAB as a surfactant for enhanced dispersion of GNPs with pH lower than 5. Figure 9 demonstrates a uniformly distributed GNPs in HA powder via the hydrothermal method, chemical precipitation, ultrasonication, and ball-milling.

The composite powder processing involves the mechanical mixing of HA and GNPs precursors. These are sometimes preceded by chemical mixing routes to enhance the GNPs dispersion. The mechanical mixing method can be divided into three parts: (i) ball milling, (ii) ultrasonication, and (iii) stirring. Ball milling is one of the most common methods for dispersing GNPs in HA for the fabrication of coated and sintered parts. Some studies reported that ultrasonication is more effective than ball milling in the synthesis of HA composites. Ultrasonication is widely utilized in the preparation of HA composite precursors for coating technique such as electrophoretic deposition. Some studies have utilized ultrasonication for the mixing of GNPs and HA to obtain uniform GNPs dispersions for the precursor and coating stages. Other researchers reported the mixing of 2.5 wt% GNPs in biphasic calcium phosphate (BCP) powders by the ball-milling process, to prepare composites via the sintering, hot pressing, and spark plasma sintering routes.

As mentioned above, a surfactant plays an important role in the dispersion of particles during the preparation process. Nanoparticles have high surface energy due to the absence of coordinated atoms, and the van der Waals forces cause them to agglomerate. A thorough study on the dispersion of GNPs shows that anionic surfactants such as sodium dodecyl sulphate (SDS), sodium dodecyl benzene sulphonate (SDBS), and CTAB produce better dispersion of GNPs in de-ionized water. A surfactant is able to disperse nanomaterials due to its unique properties, with a water-soluble group (hydrophilic) at one end and a water-insoluble group (hydrophobic) at the opposite end. The hydrophobic end of a surfactant molecule attaches to the particle surface, while the hydrophilic part extends to the water. This is followed by the formation of an electric layer as a result of the surfactant attachment. Thus, the electrostatic charges on the particle surface prevents the agglomeration of the particles. Due to the structural compatibility

Figure 9. Distribution of GNPs in HA powder mixed using (a) hydrothermal, (b) chemical precipitation method, (c) ultrasonication, and (d) ball-milling. © Elsevier. Reprinted with permission from Baradaran et al. Permission to reuse must be obtained from the rightsholder.)
of CTAB with HA particles, CTAB is able to attach on a particular side of the HA particle therefore influencing the particle growth orientation.\textsuperscript{340} Hybrid composites of HA and functionalized graphene such as GO and rGO, have been extensively examined for osteogenesis in an \textit{in-vitro} cell culture model.\textsuperscript{16,342,343} Lee et al.\textsuperscript{17,345} examined the characteristics of rGO-coated HA composites on the osteogenic differentiation of hMSCs. For this purpose, water-soluble calcium phosphate HA powder was used to prepare the HA microparticles. DI water was used to prepare a colloidal dispersion of rGO NPs: HA microparticles at a 1:1 weight ratio vigorously vortexed for 10 min. The solvent was evaporated slowly to produce the rGO-coated HA composites. In another study, Zanin et al.\textsuperscript{346} reported the direct electrodeposition of globular n-HA on rGO, and a model to explain the preferred growth orientation. The electrodeposition of n-HA on superhydrophilic-rGO films was performed in 0.042 mol L\textsuperscript{−1} Ca(NO\textsubscript{3})\textsubscript{2}.4H\textsubscript{2}O + 0.025 mol L\textsuperscript{−1} (NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4} electrolytes (pH 4.7). The superhydrophilic-rGO films was the working electrode while a high-purity platinum wire and Ag/AgCl (3 M) were the secondary and reference electrodes, respectively. The n-HA films were deposited at −2.0 V for 30 min at 70°C. In addition, Mahto et al.\textsuperscript{347} prepared GO/HA nanocomposites for adsorption applications. A stock solution (50 ml) of calcium nitrate tetrahydrate was added drop-wise to a GO suspension with stirring. This was followed by the drop-wise addition of ammonium dihydrogen phosphate (50 ml) under argon atmosphere and stirred for 1 h at 35°C. Aqueous ammonia was added at 60°C to maintain the pH at 11. The resultant dark brown dispersion was heated at 90°C for 2 h, followed by the overnight cooling to room temperature. In a different approach, we\textsuperscript{340} explored nickel-doped BCP/GNPs composites for biomedical applications. The Ni-doped HA (3 and 6 wt.%) was prepared via the continuous precipitation method at room temperature. The Ca(NO\textsubscript{3})\textsubscript{2}.4H\textsubscript{2}O and Ni(NO\textsubscript{3})\textsubscript{2}.6H\textsubscript{2}O were dissolved into a solution, followed by the drop-wise addition of (NH\textsubscript{4})\textsubscript{4}H\textsubscript{2}PO\textsubscript{4} under vigorous stirring. The pH was adjusted with the addition of ammonia, (NH\textsubscript{4})\textsubscript{2}H\textsubscript{2}PO\textsubscript{4} and nitrate solution. The precipitate was filtered and dried at 100°C for 24 h, followed by calcination at 900°C for 1 h. The precipitate was ball-milled at 300 rpm for 2 h to produce the fine powder. The appropriate amounts of GNPs and Ni6 powders were dispersed separately in DI with 1 wt% CTAB and 1 h sonication. For the fabrication of the composites, GNPs dispersions, and powders with concentrations of 0.5 wt%, 1 wt%, 1.5 wt%, and 2 wt% GNPs were sonicated for 1 h and ball milled at 400 rpm for 15 h. Finally, the slurry was dried in an oven at 90°C for 24 h. Apart from the aforementioned approaches, Raucci et al.\textsuperscript{348} prepared biomineralized HA nanocrystals–GO by biomimetic treatment and \textit{in-situ} sol–gel synthesis without the use of toxic components. A HA–GO nanocomposite was synthesized at room temperature using Ca(NO\textsubscript{3})\textsubscript{2}.4H\textsubscript{2}O and (NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4} as the Ca and P precursors, respectively. The amount of Ca(NO\textsubscript{3})\textsubscript{2}.4H\textsubscript{2}O was chosen to establish a Ca:P ratio of ca. 1.67 ± 0.5. In most cases, the molar ratio of Ca:P differs between 1.2–2 for HA and stoichiometric HA, while the Ca:P molar ratio is 1.67, although this value is different in the living organism. The Ca(NO\textsubscript{3})\textsubscript{2}.4H\textsubscript{2}O was dissolved in distilled water followed by the addition to a GO suspension to initiate the reaction between Ca\textsuperscript{2+} and GO functional groups. A homogeneous GO–Ca\textsuperscript{2+} compound was obtained via the ultrasonic dispersion, followed by the drop-wise addition of phosphorus solution (3.58 M) at pH 11. Gelation occurred only after 2 h of stirring at ambient temperature. It was observed that the different amounts of GO (1, 1.5, and 2 wt%) delayed the gelling time for 15 min.

\subsection*{2.4. Sintering and consolidation of HA–CNSs composites}

The sintering of HA composites allows the formation of dense solid shapes, useful in orthopedic applications. Sintering requires the mechanical compaction of powders followed by a firing process until diffusion combines the individual particles. The mechanical compaction and firing processes, along with the chemistry and morphology of the starting powder, determines the microstructure of the solid material. The main considerations are the control of porosity, good dispersion of powder fillers and minimal chemical dissociation of HA to other phases (i.e., TCP, CaO). The microstructure and chemical composition are the important aspects controlling the mechanical properties of ceramics.\textsuperscript{349} The consolidation of HA composites by sintering is performed after the powder compaction process, which can be done by both uniaxial and isostatic pressing. A wide range of sintering temperature between 1050–1200°C with a dwell time of 1–3 h is adopted for the HA–GNPs consolidation. However, the higher temperature and longer heat treatment time not only increase the crystallinity and density of HA, but also increases the decomposition of HA. The density of the HA–GNPs composite is lower than HA with the same sintering conditions, which suggests that GNPs prevent closure of the porosity by holding the grains matrix apart. Nevertheless, several sintering methods for ceramic densification were suggested such as pressureless sintering, hot-isostatic/hot-press sintering, microwave sintering, spark plasma sintering (SPS), and vacuum sintering.
With regards to the HA–GNPs composites, several methods of sintering have been performed to consolidate the samples. It has been reported that the BCP–GNPs composites were prepared by hot pressing (HP) of the powders at 1150°C, in a multi-purpose high temperature furnace at 30 MPa in argon for 1 h. The amounts of GNPs in the composites were 0, 0.5, 1, 1.5, 2, and 2.5 wt%. The composites had almost the same grain sizes, although prepared with different amounts of GNPs. Moreover, we examined the sintering behavior of Ni-doped BCP–GNPs composites. The as-prepared powders were pressed at 250 MPa to form discs (5 mm in diameter × 2 mm in height) and sintered at 1150°C by hot isostatic pressing (HIP) in high-purity argon at 160 MPa. On the other hand, the main advantages of the SPS method for the fabrication of HA-based composite are: (i) fine grain structure retention and (ii) decrease in HA decomposition. The SPS method is a promising technique for the fabrication of nanostructured materials, due to the grain size retention after the sintering process. This method is appropriate for ceramics, as the grain size refinement could simultaneously enhance the hardness and fracture toughness of the ceramic. The ideal SPS temperature for the HA–GNPs system is 1150°C, as lower temperatures result in poor consolidation while higher temperatures result in an excessive grain growth.

3. Properties of HA–CNSs composite and its biofunctionality

3.1. Chemical and physical properties

Generally, there are various factors that affect the decomposition of HA. The main factor is the stability of HA structure against the sintering temperature. It has been found that the decomposition temperature of HA can be drastically decreased by the (i) reduction of the water partial pressure, (ii) lowering of the CP ratio, and (iii) sintering with particulate additives. Dehydroxylation and decomposition are two steps in the thermal decomposition of HA. The dehydroxylation to oxyhydroxyapatite (OHA) usually occurs at temperatures above 900°C in air and 850°C in dry atmosphere. The thermal decomposition of HA at higher temperatures (1200–1450°C) depends on the Ca/P ratio. As the Ca/P < 1.67, HA decomposes to the β-TCP phase (α-TCP phase at higher temperatures) and TTCP. Besides, HA dissociates to CaO when the Ca/P ratio is > 1.67. The thermal decomposition of HA to TCP has two important effects: (i) the dissociated phases can cause problems in the densification and decreases the structural strength; and (ii) the unwanted phases from the decomposition of HA increases the dissolution rate in physiological pH. There is also a similar dilemma for the HA–GNPs composites. On the other hand, water released from the HA dehydroxylation could react with GNPs and damages the nanosheet. The phase dissociation of HA–GNPs composites can be analyzed by the XRD profiles. Zhang et al. detected the dissociation of HA to α-TCP in 1 wt% GNPs, due to the lower sintering temperature (1150°C) and rapid processing conditions, during the SPS process. In another study, Zhao et al. examined the XRD of the GNP–BCP composites after the hot-pressed sintering process and showed that the incorporation of GNPs has no influence on the stability of HA and β-TCP. With regards to the substituted HA, Kadir et al. observed a peak related to Zn containing β-TCP in the XRD profiles of 2.5 wt% Zn, which suggests that a small amount of β-TCP might have been formed from the addition of Zn. Further increase in the Zn content to 5 wt% led to an evident decline in the intensity of the HA peaks and a rise in the intensity of Zn-TCP peak. They revealed that the development of TCP was due to the difference in the ionic radius of Zn and Ca, which caused a distortion of the crystal structure. Recently, Šupová reported that the ease of substitution or atomic doping in apatites encourages a wide range of biomedical applications. This article presents evidences of the growing focus on ionic substitutions as a powerful method to enhance the biological properties of HA. This is through the modifications of the structural, chemical, and morphological characteristics, or even by the utilization of the therapeutic properties of the substituent ions. However, it should be stressed that very few studies have reported comprehensive in-vitro and/or in-vivo biological tests, and many studies have yet to be done on the lengthy path from fundamental studies to clinical applications. This shows that effective collaborations between materials scientists, chemists, bioengineers, biologists, and clinicians are much need to disseminate research findings into medical applications. In this regard, our recent research has shown that the structural features and phase compositions of the Ni-doped BCP–GNPs composites are strongly dependent on the dopant content and GNPs, after the calcination and sintering processes. The sintered sample showed a highly crystalline phase of monolithic HA in the absence of nickel while the addition of 6% Ni resulted in the decomposition of HA to β-TCP and CaO. Besides, the BCP–GNPs composite was formed in the presence of both 6 wt% Ni and 1.5% GNPs.

3.2. Mechanical properties

The requirements of the ceramic implants are much influenced by the role in the body. For instance, the requirements for an ear implant will be different from those of a total hip prosthesis (THP). In general, the
ceramic must fulfill two important criteria: (i) the mechanical properties must match the replaced tissues and (ii) must be stable in the physiological environment. The main advantage of ceramics materials as implants is the biocompatibility, some have specific reactions in the body while others are inert. However, the main drawbacks of most bioceramics are the low toughness which can affect the reliability, and the high Young’s modulus (E) which produces stress shielding. Stress shielding could occur if the implant has higher E than the bone and weakens the bone in the region where the applied load is the lowest or when under compression. The area of the bone which is unloaded/loaded under compression could undergo biological responses which could lead to bone resorption. Therefore, the elimination of stress shielding, by decreasing E, is the primary focus in bioceramic composites.\(^2\) The primary focus of composite fabrication is the improvement of the mechanical properties, especially the toughness. For bioceramic composites, the main goal is to decrease E and increase \(K_{IC}\). The earliest bioceramic was a stainless-steel fiber-glass composite, made from AISI 316L stainless steel and Bioglass® 45S5. One of the main problems with these types of composites is the mismatch in \(\alpha\) of the two components. The HA-reinforced polyethylene is an excellent example of a composite which possesses properties not found in the pure material. These bone replacement composites are ductile, bioactive, and have a matched modulus. However, when the volume fraction of HA increases to more than 0.45, the fracture mode changes from ductile to brittle.\(^3\) In this regard, we\(^4\) recently explored the effects of the dopant and GNPs content on the mechanical properties of Ni-doped BCP–GNPs composites. The high specific surface area of GNPs is capable to form and wrap around the HA grains and increases the contact area with the matrix. Therefore, the bonding strength of the GNPs and HA grains could be enhanced significantly, and more energy is needed for the nanofiller pull-out from the matrix. The wrinkled and rough surface of the GNPs also plays a key role in increasing the mechanical interlocking, which could lead to an increased load-transfer efficiency between the HA matrix and GNPs.\(^5\) A comprehensive summary of the effect of GNPs and metal ions substitution on the elastic modulus, fracture toughness, and hardness of the HA–CNSS composites are presented in the following subsections.

### 3.2.1. Fracture toughness

Bone is the load-support parts of a vertebrate, therefore must possess good fracture toughness (\(K_{IC}\)) to prevent fracture and cracking upon high cyclic loading during limb movements. The fracture toughness of the cortical bone (2 MPa.m\(^{0.5}\)) is reported to be larger than dense HA (1 MPa.m\(^{0.5}\)). The main goal in the fabrication of the ceramic–matrix composites (CMCs) is the enhancement in the fracture toughness of the material, or the resistance against fracture when a crack is present. The lower tension strength compared to compression is an inherent problem with brittle ceramics. The enhancement in the toughness normally improves the strength and stiffness of the composites.\(^35\) GNPs and ionic substitutions in HA have been utilized to improve the fracture toughness of HA-based composites. These reinforcements are able to impede crack growth in a ceramic by several approaches (Figure 10), such as: (i) crack tip deflection, when cracks propagate through a matrix and reaches a GNP, the crack is deflected while absorbing energy and results in the toughening of the matrix; (ii) pull-out, or the pulling out of GNPs from the matrix, this could dissipate energy and leads to toughening; (iii) crack bridging, GNPs are bridges which could prevent the widening of cracks as the GNPs bridges require more energy to open the cracks; and (iv) crack branching, this toughening mechanism can be frequently observed in polycrystalline-graphene composites which originates from the interaction of the cracks and smaller size GNPs.\(^34\)\(^,\)\(^35\)

Most studies have employed indentation-based method to measure the fracture toughness. Previous reports mentioned that the value of \(K_{IC}\) in each study depends on several aspects such as the powder morphology, processing route, and structure type, i.e., free standing or coating.\(^19\)\(^,\)\(^35\)\(^9\) The percentage improvement of \(K_{IC}\) in the HA–GNPs composite is calculated by referring to the \(K_{IC}\) value of HA in the absence of GNPs. The highest improvement in \(K_{IC}\) is \(\sim\)80% was obtained by Zhang et al.\(^20\) with 1 wt% GNPs addition sintered by the SPS method. Liu et al.\(^36\) reported a fracture toughness of 3.94 MPa m\(^{1/2}\) for a HA–rGO nano composite, an increase of 203% compared to the pure HA. This is due to the presence of crack deflection, crack bridging and crack tip shielding at the HA–rGO interfaces which strengthen the composites. Zhao et al.\(^34\)\(^3\) reported that the mechanical properties of the GNPs–BCP composite measured parallel to the direction of the hot pressing (HP) method were higher compared to the measurement along the perpendicular direction. The 1.5 wt% GNPs composite gave a maximum bending strength and fracture toughness of 151.82 MPa and 1.74 MPa m\(^{1/2}\), respectively, when measured parallel to the HP direction. These are around 55% and 76% higher than the monolithic BCP, respectively. Mehrali et al.\(^36\)\(^1\)\(^,\)\(^36\)\(^2\) also investigated the increase in the fracture toughness of calcium silicate fabricated by the HIP method in the presence of 1.5% GNPs. With regards to the substituted HA, few studies have investigated the effect of metal ion doping on the fracture toughness. Basar et al.\(^37\) reported the fracture toughness of pure, yttrium
(Y), and fluoride (F) doped HA vs. the sintering temperature. A maximum fracture toughness of 2.1 MPa m$^{1/2}$ was obtained for the 2.5YFHA sample sintered at 1100°C. Our latest studies on Ni-doped BCP–GNPs composites showed that the fracture toughness of Ni6 and 1.5Ni6 increased by 59% and 163%, respectively, compared to monolithic HA, where the toughening mechanisms such as crack deflection, graphene necking, crack bridging, crack branching, and pull out were detected as shown in Figure 11.340

Figure 10. Toughening mechanisms in HA–GNPs composites: (a) crack deflection, (b) pull-out, (c) crack bridging, and (d) crack branching. (© Elsevier. Reprinted with permission from Baradaran et al.340 Permission to reuse must be obtained from the rightsholder.)

Figure 11. (a) The mechanical properties and relative density of Ni-doped BCP–GNP composites compared to pure HA, (b) $E$, (c) microhardness, and (d) fracture toughness.
3.2.2. Elastic modulus (E)

The elastic modulus of the human cortical bone is between 15–25 GPa, and is much higher (80–120 GPa) than the consolidated HA. The mismatch of E at the bone-implant interface could risk the delamination or fracture of the implant. But, HA establishes a strong bond at the HA-bone interface, which decreases the fracture and delamination. Thus, the elastic modulus of HA need not to be improved before incorporation into the bone, unlike the fracture toughness. However, the increase in E directly affects the fracture toughness of the ceramic composites. The improvement of the elastic modulus in HA–GNPs composites is due to the higher elastic modulus of GNPs (1TPa) and excellent bonding at the HA/GNPs interface. The HA–GNPs matrix starts to deform due to the lower elastic modulus upon the application of stress. The effective transfer of stress from the matrix to the reinforced parts could occur if the GNPs has a strong interfacial bonding with the HA matrix. GNPs have the ability to absorb more stress than HA, resulting in an enhanced elastic modulus of the HA–GNPs composites. Many studies have reported an increase of E up to 70% with a GNPs content between 0.25–2.5 wt%. The increase of E in the composite is due to the homogeneous dispersion and good interfacial bonding of GNPs. Zhang et al. measured the elastic modulus for 1 wt% HA–GNPs composite and showed improvements of 40%, while Li et al. prepared GO–HA and chitosan (CS)–GO–HA nanocomposites and found that the elastic modulus was increased from 5.55 to 19.09 GPa due to the reinforced effects of GO on HA. Jankovic et al. also reported the electrophotore deposition of HA–GNPs composite on Ti with increased elastic modulus of almost 50%. Liu et al. also reported that the elastic modulus of HA–rGO SPS pellets increased with the increase in the rGO content, while the E of the HA–RGO composite was augmented by 47.6% with the increase in the rGO content. With regards to the substituted HA, there are few reports which investigated the effect of different ions on the elastic modulus of the composites. Yatongchai et al. investigated the effects of strontium (Sr) additions (5 and 10%) on the Weibull statistics and phase assemblage of HA. They reported that the porosity in the undoped HA sample is relatively independent from the Weibull modulus. The 5% Sr-HA sample showed a slight increase in the Weibull modulus while the 10% Sr-HA sample gave the highest Weibull modulus value for all sintering temperatures. Xu et al. found that the E value of a sintered spray dried HA increased with an increase in the silica doping from 1 to 5 wt%, while the E values increased from 84 to 100 GPa (15%) compared to pure HA (~89 GPa). With regards to the Ni-doped BCP–GNPs composites, our results indicated that E increased by 120% and 85% for Ni6 (6 wt% Ni) and 1.5Ni6 (6 wt% Ni and 1.5% GNPs), respectively, compared to the monolithic HA (Figure 11b).

3.2.3. Hardness

The addition of CNSs such as CNTs and GNPs influences the hardness of HA-based composite. The plastic deformation in the HA matrix could be decreased by the greater stiffness of CNSs which provides a strengthening effect. The structural refinement and grain boundary pinning by CNSs also increase the fracture toughness and hardness simultaneously. The Vickers’ hardness and nano-indentation are the most commonly used technique to quantify the hardness of the HA–CNSs composites. It has been reported that the absolute hardness values differ between the nano-indentation and Vickers techniques, due to the wide differences in the tip geometry, applied load, and measurement length scale. The main advantage of the micro-indentation test is that a much larger sample volume can be indented and a higher volume fraction of the GNPs is encountered compared to the nano-indentation test. With regards to the HA–GNPs, Zhao et al. reported that the Vickers hardness for 1 wt% GNPs–HA composite showed 30% improvements compared to pure HA, due to the good bonding strength between the GNPs and HA grains which significantly enhance the hardness. Li et al. found that the hardness of GO-HA increased from 367.59 ± 25.76 to 624.32 ± 11.77 MPa, due to the interaction of the HA nanoparticles and GO matrix. The HA hardness increases with the addition of GNPs due to the strengthening of the matrix and grain size refinement, both of which hinders the plastic deformation. However, the weak interface and agglomeration of the GNPs could decrease the hardness of the composite. With regards to the substituted HA, Kalita et al. performed Vickers hardness test on pure and doped nanocrystalline sintered HA structures. They showed that the surface hardness of nanocrystalline HA ceramics is dependent on the presence of zinc and magnesium dopants. Basar et al. reported the co-substitutions of Y³⁺ (2.5, 5, and 7.5 mol%) and F⁻ (2.5 mol%) ions in doped HA and their effects on the microhardness. The sample sintered at 1300°C gave the largest microhardness value of 5.9 GPa compared to the pure HA with 4.5 GPa. In another study, Xu et al. investigated the effect of silica-doped HA on the decrease of hardness value of the bulk samples compared to pure HA. The relatively lower hardness value of the doped samples was due to the lower bulk density of the material. The formation of pores and the phase transformation of HA into β-TCP resulted in lower densities.
the hardness value increased gradually with the increase of La$^{3+}$ incorporation by 14% for L5HA (50 mM La$^{3+}$). With regards to the Ni-doped BCP–GNP composites, our results showed that the hardness increased by 55% and 59% for Ni6 (6 wt% Ni) and 1.5Ni6 (6 wt% Ni and 1.5% GNP), respectively, compared to the monolithic HA$^{340}$ (Figure 11c).

### 3.3. In-vitro bio-functional performances

Recent studies have revealed that graphene and graphene composites have several advantages, for instance, excellent antibacterial property for the proliferation and adhesion of osteoblasts, non-toxicity against human osteoblasts and the ability of apatite mineralization.$^{252}$ The apatite precipitation capability of a surface during immersion in simulated body fluid (SBF) is an indicator of the in-vivo integration ability with the new bone. The validity of the SBF immersion test as an indicator of bio-compatibility is justified from a statement by Kokubo (the originator of SBF) and Takadama, “apatite formation on a material in SBF is useful for predicting the in-vivo bone bioactivity of a material.”$^{46}$ Furthermore, a new bone formation on an orthopedic surface is strongly dependent on the bone cell (osteoblast) attachment, differentiation and proliferation.$^{369}$ Thus, the behavior of osteoblast determines the orthopedic surface biocompatibility. It is proven that the GNP composites are suitable for bone-like apatite generation. The mineralization process occurs in three stages: (i) dissolution, (ii) precipitation, and (iii) apatite formation stages. In the first step, the dissolution of calcium and phosphate ions takes place.$^{14}$ Nevertheless, the process and kinetics of HA deposition on the composite are influenced by the surface area and negative surface charges. To examine the potential applications of GO as orthopedic implants in bone rehabilitation, some researchers have focused on the fabrication and cell behavior of GO on quartz substrate. The formation of a HA layer on GO coated substrate in SBF strongly indicates the bioactivity of the coated substrate.$^{370}$ The MC3T3-E1 cells were cultured on GO coated substrate, and the apoptosis, viability, cytotoxicity, and proliferation were absent from the GO coated substrate. However, the GO coatings enhanced the cell differentiation compared to the tissue culture plates and non-coated substrate, from the higher levels of osteocalcin (OC) secretion and alkaline phosphatase (ALP) activity on the coated substrate. They reported that the GO coatings enhanced MC3T3-E1 cell differentiation and showed good biocompatibility, which could be an excellent material for orthopedic implants. Mohandes et al.$^{371}$ prepared a new chitosan/GO/HA nanocomposite which showed higher bioactivity than pure HA. Janković et al.$^{372}$ prepared a graphene based Ag/HA/Gr composite on titanium by electrophoretic deposition (EPD) with improved corrosion resistance in SBF. The enhanced bioactivity of the Ag/HA/Gr composite was evident with the formation of a stable apatite layer in SBF. The antibacterial activity against Escherichia coli and Staphylococcus aureus, and non-cytotoxicity towards peripheral blood mononuclear cells (PBMC), suggest a bright prospect in biomedical applications. With regards to the HA–GNPs composites, there are some reports on the apatite formability in SBF. Zhang et al.$^{20}$ investigated the use of GNPs as reinforcements to HA for load-supporting orthopedic applications together with the in-vitro biocompatibility. The in-vitro osteoblast growth tests showed that the GNPs addition improved the apatite mineralization and osteoblast adhesion. It is also suggested that the GNPs provided osteoblast adhesion by forming more nucleation sites, to facilitate the apatite mineralization. In a similar work,$^{338}$ HA was successfully grafted to GNPs and investigated for the differentiation of human fetal osteoblastic cell line (hFOB 1.19). The results showed that the hFOB 1.19 cells differentiated and proliferate in the graphene-HA media. Fan et al.$^{317}$ showed a gradual formation of a dense and thick apatite layer on the surface of HA/GNPs composite immersed in SBF for 7 days. With regards to the substituted HA, Joshy et al.$^{368}$ investigated the bioactivity of La-doped HA by immersion in SBF solution. After the immersion in SBF, the HA crystal plate formation was followed by the conversion to spherical HA deposition, due to the bioactivity. Medvesky et al.$^{152}$ also reported that a Mn content up to 0.25 wt% in HA did not influence the growth rate of the HA particles in SBF, but a rapid decrease in the growth rate of HA in SBF was due to the higher concentration of Mn on the HA surface, after the ion-exchange process. In addition, Cox et al.$^{373}$ confirmed the growth of an apatite layer on the pure HA surface, Sr-, Mg-, and Zn-doped HA samples in SBF after 28 days. Moreover, the formation of bone-apatite observed on the Ag-containing HA coating during the immersion in SBF suggests a favorable biological response of the product. The presence of silver ions in the SBF did not affect the formation of bone-apatite on the Ag-containing HA.$^{374}$ With regards to the Ni-doped BCP–GNP composite, the EDS results demonstrated the formation of a layer similar to apatite on the pure HA surface after 7 days of immersion in SBF. In Ni6, tiny spherical apatite crystals precipitated on the sample separately but without full surface coverage. The 1.5Ni6 composite showed well covered apatite in the presence of GNPs just after 5 days immersion in SBF (Figure 12).$^{340}$

On the whole, the HA–GNPs composites meet almost all requirements as an excellent implant coating material
with good biocompatibility, excellent tribological, and mechanical strength. These unique characteristics of HA–GNPs composites could initiate broad applications in bone graft materials in the future.

4. Conclusion and outlook

This article provides a systematic review on how the physicochemical features, mechanical behavior and in-vitro bio-functional performances of HA is affected by CNSs particularly GNPs. In fact, this article gives the current state of knowledge of the preparation and characterization of HA–GNPs nanocomposites. It is obvious that graphene and its nanostructural derivatives have substantial potential as fillers for ceramic matrices, which is confirmed by the considerable improvements in the mechanical and biological properties reported in a number of publications. As described throughout this article, ionic substitutions and GNPs can fulfill the majority of the necessary requirements of an effective bone graft substitute. These reinforcements in HA-based ceramics impede crack growth by deflecting crack tips, pull-out, crack bridging, and crack branching. The results of our recent studies also showed that the fracture

Figure 12. FESEM images of apatite formation and morphology of the osteoblasts cultured on (a,d) monolithic HA, (b,e) Ni6, and (c,f) 1.5Ni6. (© Elsevier. Reprinted with permission from Baradaran et al. Permission to reuse must be obtained from the rightsholder.)
toughness of substituted BCP and its composites with GNPs increased by 59% and 163%, respectively, compared to monolithic HA. Moreover, the formation of bone-like apatites on substituted BCP–GNPs composites during immersion in SBF suggests a favorable biological response of the composites. However, work on HA–CNSs composites is still in the early stages and there are significant efforts that are required to be carried out to improve their efficiency.

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