



# An overview of graphene-based hydroxyapatite composites for orthopedic applications

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## ABSTRACT

Hydroxyapatite (HA) is an attractive bioceramic for hard tissue repair and regeneration due to its physicochemical similarities to natural apatite. However, its low fracture toughness, poor tensile strength and weak wear resistance become major obstacles for potential clinical applications. One promising method to tackle with these problems is exploiting graphene and its derivatives (graphene oxide and reduced graphene oxide) as nanoscale reinforcement fillers to fabricate graphene-based hydroxyapatite composites in the form of powders, coatings and scaffolds. The last few years witnessed increasing numbers of studies on the preparation, mechanical and biological evaluations of these novel materials. Herein, various preparation techniques, mechanical behaviors and toughen mechanism, the *in vitro*/*in vivo* biocompatible analysis, antibacterial properties of the graphene-based HA composites are presented in this review.

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## 1. Introduction

Bone tissues consist of organic and inorganic components, with self-healing ability and great capability to withstand mechanical loading. Fixation of bone fractures and non-unions, correction of spinal deformities and replacement of arthritic joints [1] are major unmet clinical needs. Traditionally, biological approaches for bone repair involve using autografts and allografts of cancellous bone [2]. Nowadays, calcium phosphate ceramics and bioactive glasses are introduced as promising osteoinductive and osteoconductive substitutes for large orthopedic defect remolding or regeneration [3]. In addition, these bioceramics are also utilized as coating on metallic implants to provide long-term performance of the devices and to minimize micromotion between bones and implants during

physiologic loading [1,4].

Hydroxyapatite (HA,  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ ) possesses chemical and crystallographic similarities to inorganic components of the bone matrix and the teeth [5] with excellent osteoconductivity and osteoinductivity. It has been clinically used as bioactive coatings on dental and orthopedic implants, enabling the adhesion and proliferation of osteoblast cells on the prosthetic surface, and resulting in biological fixation between bone tissues and the implant [6]. However, one primary limitation, when used under major load bearing, is its poor mechanical properties, such as low fracture toughness and tensile strength [5]. To address this problem, specific reinforcing materials, such as carbon nanotubes [7], polyethylene [8],  $\text{Al}_2\text{O}_3$  and  $\text{TiO}_2$  [9], are typically used to prepare HA composites with increased mechanical properties, but these materials result in significantly less bioactivity than that of pure HA.

Graphene is rapidly rising as a promising material for biomedical applications [10,11], featuring two-dimensional nanosheet of hexagonally bonded carbon atoms, with large surface area, high conductivity, strong mechanical properties and good

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biocompatibility. The graphene-based composites have great advantages when used in bone repair or regeneration, as it can induce osteogenic [12–14] and chondrogenic [15] differentiation of stem cells. Compared with other reinforcement fillers, graphene can greatly increase the mechanical properties of the composite at low content, and its high elasticity and flexibility (adaptability to flat or irregular surfaces) also renders graphene and its derivatives (graphene oxide (GO) and reduced graphene oxide (rGO)) as promising mechanical fillers for biomaterials.

Recently, biomaterial scientists have explored the possibilities of preparing graphene-based HA composite for orthopedic applications with increased bioactivities and mechanical properties. Graphene-based HA composites can be prepared in the form of powders, bulks, coatings and scaffolds. The powders or bulk composites can be used to repair the bone defects or small non-unions. This novel material can also be coated onto orthopedics metallic implant to increase its bone-binding abilities. As for the large defects or bone loss, three dimensional porous graphene-based HA composites can be incorporated into the damaged hard tissues to accelerate their regeneration.

The related research has begun very recently in 2009 [16]. The chronological tendency of the research papers on graphene/HA system is shown in Fig. 1, showing an increasing interest in this area. A significant growth in the number of publications from 2013 to 2016 highlights the novelty and importance of this topic in the up-to-date scientific community. Therefore, in the foreseeable future, more and more related works will be undertaken, and it is the right time to present a comprehensive review of current achievements and findings in this field, which may provide guidance and future directions for further study.

Herein, we present a comprehensive review on almost all of the available investigations on graphene/HA system. Articles were identified via Web of Science and Google Scholar by searching “graphene” and “hydroxyapatite” which were published up to June 2017. This review paper includes the issues regarding the preparation methods, mechanical properties, *in vitro* and *in vivo* biocompatibility of graphene/HA composites, as well as the underlying challenges required to be coped with.

## 2. Preparation of composites

Various preparation techniques could be exploited to make this

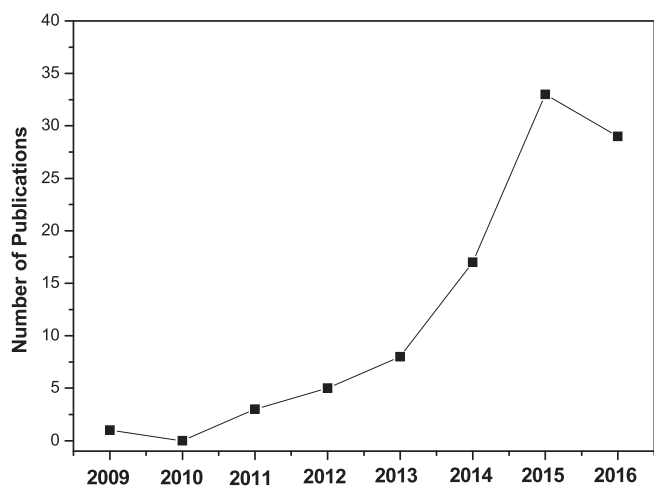


Fig. 1. The number of publications on Graphene-based HA composites from year 2009–2016 (2009 [16], 2011 [17–19], 2012 [20–24], 2013 [25–32], 2014 [33–49], 2015 [50–82], 2016 [83–112]). This figure does not include the papers published in 2017 [113–130].

interesting composites, which is summarized in Fig. 2. In most cases, the composite prepared under high temperature or high pressures have high crystallinity and mechanical properties, such as hydrothermal synthesis, spark plasma sintering and hot isostatic sintering. However, thermal spraying techniques usually lower the crystallinity of the HA coating. HA could be synthesized onto graphene and its derivatives and be directly mixed with these nanofillers by ultrasonic dispersion and ball milling.

### 2.1. Graphene/HA composite powder

#### 2.1.1. *In situ* synthesis

Nano HA particles are successfully fabricated on GO [30], chitosan functionalized GO [30] and rGO [29] surfaces using *in situ* synthesis methods. Usually, as shown in Fig. 3a, graphene-based powders are first dissolved and exfoliated in DI water by ultrasonic dispersion to obtain a uniform solution; then  $\text{Ca}(\text{NO}_3)_2$  is added into the graphene-based solutions by stirring for a desired time; afterwards, the pH of the suspension is adjusted to 9–10 using ammonia water, and  $(\text{NH}_4)_2\text{HPO}_4$  was added dropwise into the mixture [30]. The resulting composite solutions are recommended to be aged for days to ensure the fully transformation of apatite into hydroxyapatite with good phase purity and well crystallinity. During the synthesis, the oxygen-containing functional groups on GO surfaces behave as receptor sites for  $\text{Ca}^{2+}$  through electrostatic interactions; these anchored cations can *in situ* react with the phosphate ions to obtain apatite nanoparticles. The underlying reaction mechanism has been proposed and discussed by Li et al. [30]; the distribution and the microstructures of HA on graphene are mainly influenced by (1) the amounts and types of the oxygenous groups on the graphene-based templates and (2) the concentration of the reagents ( $\text{Ca}^{2+}$  and  $\text{HPO}_4^{2-}$ ), solution pH values and so on. Besides,  $\text{Ca}(\text{OH})_2$  and  $\text{H}_3\text{PO}_4$  are also utilized by Gururaj et al. to *in situ* deposit HA on rGO nanosheets [29].

Composite, prepared in this method, is expected to increase the interfacial bonding strength between graphene and HA, facilitating the stress transfer from the matrix to the graphene-based nanofillers. This facile approach is economical and can be industrially mass-produced.

#### 2.1.2. Biomimetic mineralization

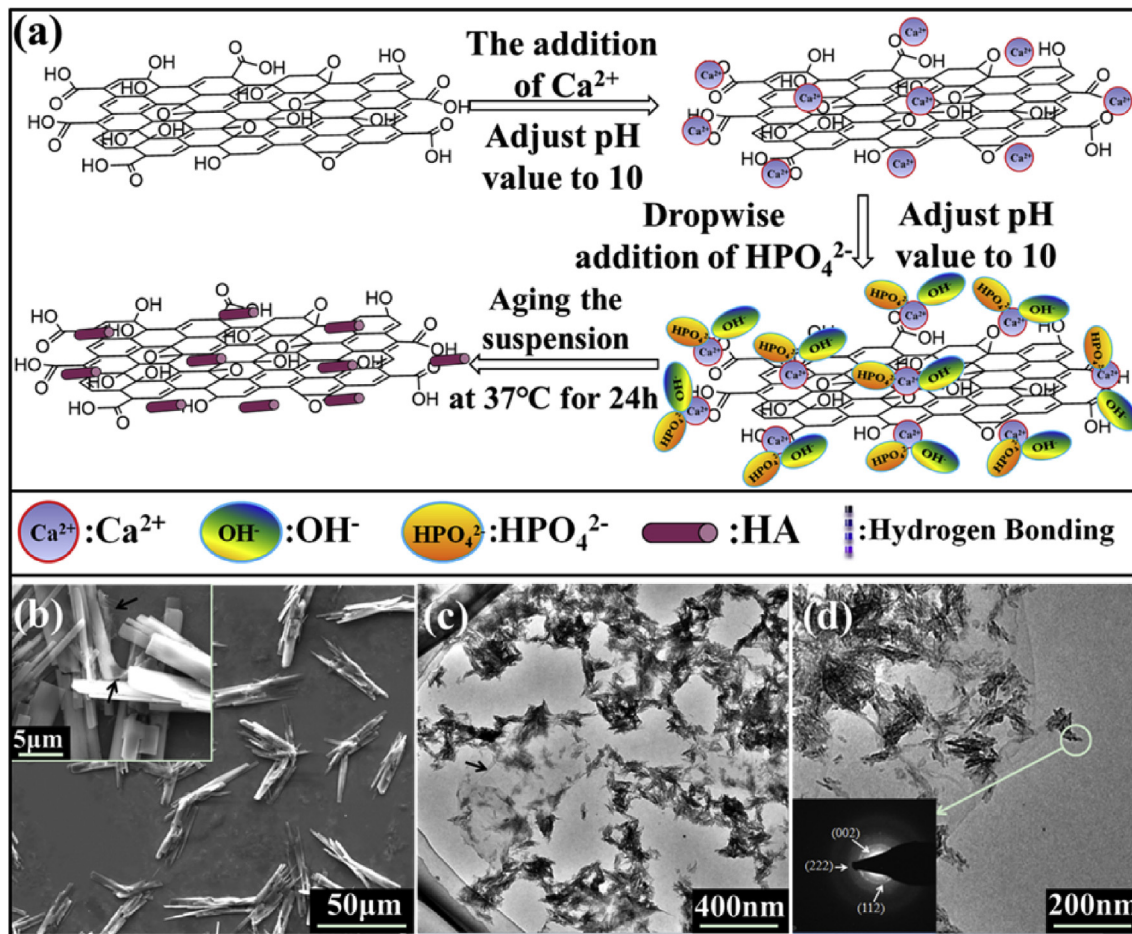
Biomimetic mineralization is a facile and environmental friendly method to synthesis bone-like apatite under ambient conditions in aqueous environments. Usually graphene and its derivatives are immersed in a supersaturated or unstable solution with calcium ions and phosphate ions concentrations similar to simulated physiological condition, and apatite will nucleate and precipitate on the surface of those graphene-based materials. During the mineralization process, GO greatly enhance the nucleation and crystallization of HA, resulting in a hybrid homogeneous GO/HA coatings with dense and fine flake-like HA nanocrystalline [54]. Usually, graphene and its derivatives are surface-functionalized by bioactive materials to endow the composite with novel properties and facilitate the biomimetic deposition of HA. The GO can be modified by gelatin to mimic the charged proteins in extracellular matrix for regulating bone formation, and the presence of gelatin improves the attraction of calcium ions and promotes the nucleation of HA [37]. Besides GO can be also bio-functionalized by polydopamine [24], casein phosphopeptide [26], carrageenan [35], chitosan [104,131], fibrinogen [33] or peptide [78] to improve the mineralization process.

#### 2.1.3. Hydrothermal synthesis

Hydrothermal synthesis of graphene/HA composite involves of dispersing graphene or GO into aqueous solutions containing

Forms	Technique	Process	Advantages (+) and Disadvantages (-)	Ref.
Powder	In situ synthesis	1) Graphene-based powders dissolved in water 2) Combined with Ca ions by continuously stirring 3) (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> was gradually dropwised 4) Keep the pH of the suspension to 9~10 5) Aging the suspension for desired time	(+) Good phase purity (+) Increased the interfacial bonding strength (+) The method is economic and can be industrially mass-produced (-) Low crystallinity (-) Long time for aging process	[16, 29, 30]
	Hydrothermal synthesis	1) Dispersing the nanosheets into aqueous solutions containing calcium and phosphate ions 2) Reaction under high temperatures and vapor pressures 3) Add organic substances into the system to modulate the HA morphology	(+) Good crystallinity and well-controlled stoichiometric composition (+) Suitable for large-scale production (+) Can reduce GO to rGO without using reducing agent (-) Using expensive autoclaves (-) Impossibility of observing the crystal as it grows (-) Using poisonous solvents in some cases	[46]
	Biomimetic mineralization	1) Graphene-based powders were decorated by bioactive substances 2) The powders were immersed in SBF 3) Aging the suspension for desired time	(+) Bone-like apatite (+) Increased osteogenic activities (+) Facile and environmental friendly method (-) Low crystallinity (-) Long time for aging process	[24] [33, 37, 45, 82]
	Chemical vapor deposition	1) Au nanoclusters dispersed over HA particles 2) Acetylene and methane as the carbon sources 3) Using radio-frequency chemical vapor deposition	(+) High graphene purity and large graphene sheets (-) Large and expensive equipment	[18, 72]
Bulk	Spark plasma sintering	1) Mixing HA and graphene sheets together using mechanical milling/ultrasonic dispersion/ liquid precipitation method 2) Sintering low temperatures for short periods in the SPS chamber	(+) High HA crystallinity (+) Increased mechanical properties (+) Retaining fine grain sizes (-) The use of special equipment	[19, 25, 26, 32, 79]
	Hot isostatic pressing	1) Mixing HA and graphene sheets together using mechanical milling/ultrasonic dispersion/ liquid precipitation method 2) Sintering at high temperatures under high pressure	(+) High HA crystallinity (+) ultrafine microstructures (+) Significantly improved mechanical properties (-) The use of special equipment	[31, 44, 56]
Coating	Electrophoretic deposition	1) Disperse the HA/graphene in organic solvent 2) Deposit the particles onto substrate under DC electric field 3) Post-heating treatment	(+) Good stoichiometry control, (+) High deposition rate and throughput, controllable coating thickness (+) Desirable coating uniformity and large-area deposition (-) Poor coating adhesion strength	[27, 38, 53, 67]
	Electrochemical deposition	1) Dissolving calcium and phosphate ions in a buffer solution 2) Add graphene into the suspension 3) Controlled pH values and temperature 4) Synthesis composite coating on the substrate surface	(+) Good stoichiometry control and controllable coating thickness (+) Desirable coating uniformity and large-area deposition (-) Poor coating adhesion strength (-) Low crystallinity	[28, 71, 106, 112]
	Thermal Spray	1) Add graphene into the solution 2) Prepare HA using wet chemical synthesis 3) Being spray coated onto the substrate	(+) Controllable coating thickness (+) Desirable coating uniformity and large-area deposition (+) Strong adhesion strength (-) The use special equipment	[40]
Scaffold	Lyophilisation	1) The graphene/HA mixture were mixed with polymers 2) Freezing the solution 3) Then reducing the surrounding pressure to enable the sublimation of frozen water	(+) Controllable scaffold composition (+) High porosity and connectivity (+) Large pore size (-) The use special equipment (-) Time-consume for the sublimation of frozen water	[36, 68, 76]
	Electrospinning	1) The graphene/HA mixture were dispersed in organic 2) The mixture were subjected to high voltage and being forced out from the spinneret	(+) High porosity and connectivity (+) Controllable fiber diameters (-) The use special equipment with high voltage (-) The use organic solvent is not environmental friendly	[21, 23]
	3D Printing	1) Disperse graphene/HA into specific organic solution (PVA) 2) Using 3D printer to prepare 3D scaffold	(+) Controllable porosity and connectivity (+) Can be manufactured into customized macroscopic shapes (-) The use of special equipment	[52, 120]
	Self-assembling	1) Disperse GO /HA into aqueous solution 2) heated for hours to enable the self-assembling process	(+) Controllable porosity and connectivity (+) Without using reductant to obtain rGO (-) Weak mechanical properties	[66, 122]

Fig. 2. The summary of different preparation methods of the graphene-based composites.



**Fig. 3.** (a) The proposed *in situ* synthesis mechanism of HA on pristine GO sheets. The SEM and TEM images of GO–HA (b–d) composites, the insets of insets (d) show the selected area electron diffraction (SAED) patterns of the corresponding composites. The black arrows of (b) point to the wrinkles of the GO sheets. These figures were adapted from Ref. [30].

calcium and phosphate ions, and crystallizing HA nanoparticles at high reaction temperatures and vapor pressures. Rod-like HA, with an average length of 55 nm and diameter of 13 nm, has been successfully synthesized on both sides of graphene nanosheets by using the convenient one-pot hydrothermal synthesis strategy [46]. This technique can improve the crystallinity of HA and partially reduce GO to rGO [64]. By using a mixed solvent system of ethylene glycol, N,N-dimethylformamide (DMF) and water, Baradaran et al. [44] synthesized HA nanotubes on rGO without using reducing agents. This technique is suitable for large-scale production of graphene/HA composites with good crystallinity and well-controlled stoichiometric composition, but possesses the disadvantages of using expensive autoclaves, and the inability to observe the crystal as it grows.

#### 2.1.4. Chemical vapor deposition

Chemical vapor deposition is a low cost and scalable technique to prepare graphene films [132]. Novel multicomponent and biocompatible graphene/HA/Au nanocomposites are prepared by using radio-frequency chemical vapor deposition (rf-CVD), with acetylene and methane as the carbon sources [18]. During the deposition process, Au nanoclusters are uniformly dispersed over HA particles with diameters of 2 nm ~ 7 nm and act as catalyst for graphene synthesis [18]. This research indicates that longer rf-CVD time can result in few-layers graphene with larger dimensions [18,72].

## 2.2. Graphene/HA composite bulk

### 2.2.1. Spark plasma sintering

The applications of bulk HA for hard tissue implants are limited by the low mechanical strength of consolidated HA [133]. During the conventional sintering process, HA will dissociate into tricalcium phosphate and tetracalcium phosphate at  $1000^\circ\text{C}$ – $1300^\circ\text{C}$ , and usually the high temperatures and long sintering time can cause grain coarsening behavior, which may deteriorate the mechanical properties of HA [133]. As an alternative method, spark plasma sintering (SPS) is an effective approach for preparing novel nanoceramics at low temperatures for short periods of time, with the advantages of retaining fine grain sizes. Graphene/HA composites are successfully fabricated by SPS. The starting powders used for SPS can be (i) prepared by mixing HA powders/nanoparticles and graphene sheets together using mechanical milling [79] and ultrasonic dispersion [19,25,59] and (ii) synthesized by a liquid precipitation method [32]. Graphene with diameters of several micrometers are uniformly dispersed and embedded within the HA matrix and located between the HA crystal grain boundaries without agglomerations [19]. Typical SEM images of the SPS samples were shown in Fig. 4 [25].

### 2.2.2. Hot isostatic pressing

Hot isostatic pressing (HIP) is a traditional technique to densify presintered components, consolidate powders and increase



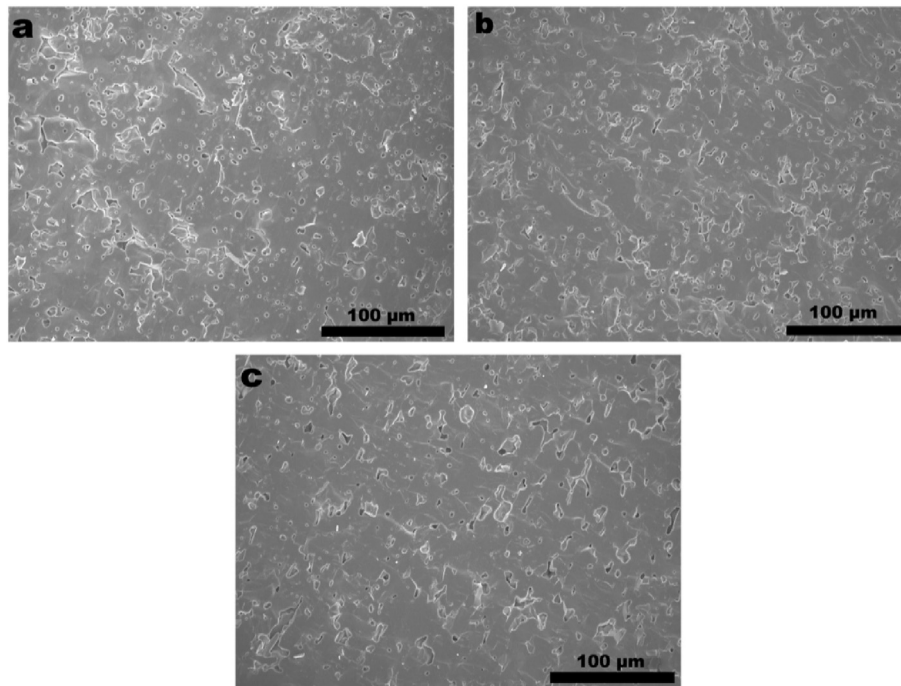


Fig. 4. SEM images of the surfaces of (a) HA, (b) 0.5 wt% graphene/HA composite and (c) 1.0 wt% graphene/HA composite [25].

interfacial bonding [134]. It can be exploited to make HA ceramics with ultrafine microstructures and significantly improved mechanical properties [135]. Recently, graphene is introduced into this system as an effective additive for toughening ceramics/composites; novel graphene/biphasic calcium phosphate composite [31], graphene/nickel-doped biphasic calcium phosphate composite [56], and reduced graphene oxide/nanotube hydroxyapatite composite [44] have been successfully fabricated by using HIP technique.

### 2.3. Graphene/HA composite coating

#### 2.3.1. Electrophoretic deposition

Electrophoretic deposition is a well-developed and aqueous-based colloidal process to deposit charged nano/micro particles onto conductive substrates under DC electric field. It has been widely used to prepare functional bioactive coatings with advanced nanostructures for biomedical applications, such as HA, bioglass and other bioceramic coatings [136]. EPD also enables the fabrication of graphene-based novel composite coatings [137]. In our previous study, GO/HA was successfully prepared on Ti substrate using cathodic electrophoretic deposition [38], as shown in Fig. 5 (a). During the coating process, GO can be considered as amphiphilic macromolecules with an edge-to-center distribution of hydrophilic (oxygenated regions and sheet edges) and hydrophobic (graphenic  $sp^2$  hybridized carbon plane) domains [138] to enhance the uniform distribution of HA in the deposition suspensions (Fig. 5 (b)). The 2D novel nanostructure of GO can increase the interlocking of HA nanoparticles and decrease the cracking and delamination the coatings [38]. EPD of HA is usually followed by post-heating treatment to increase the coating/substrate bonding strength and the addition of GO can effectively increase this cohesive strength. The microstructure of the GO/HA composite coating is proposed to be combination of an inner compact layer and outer relatively porous and unsealed layer [38].

#### 2.3.2. Electrochemical deposition

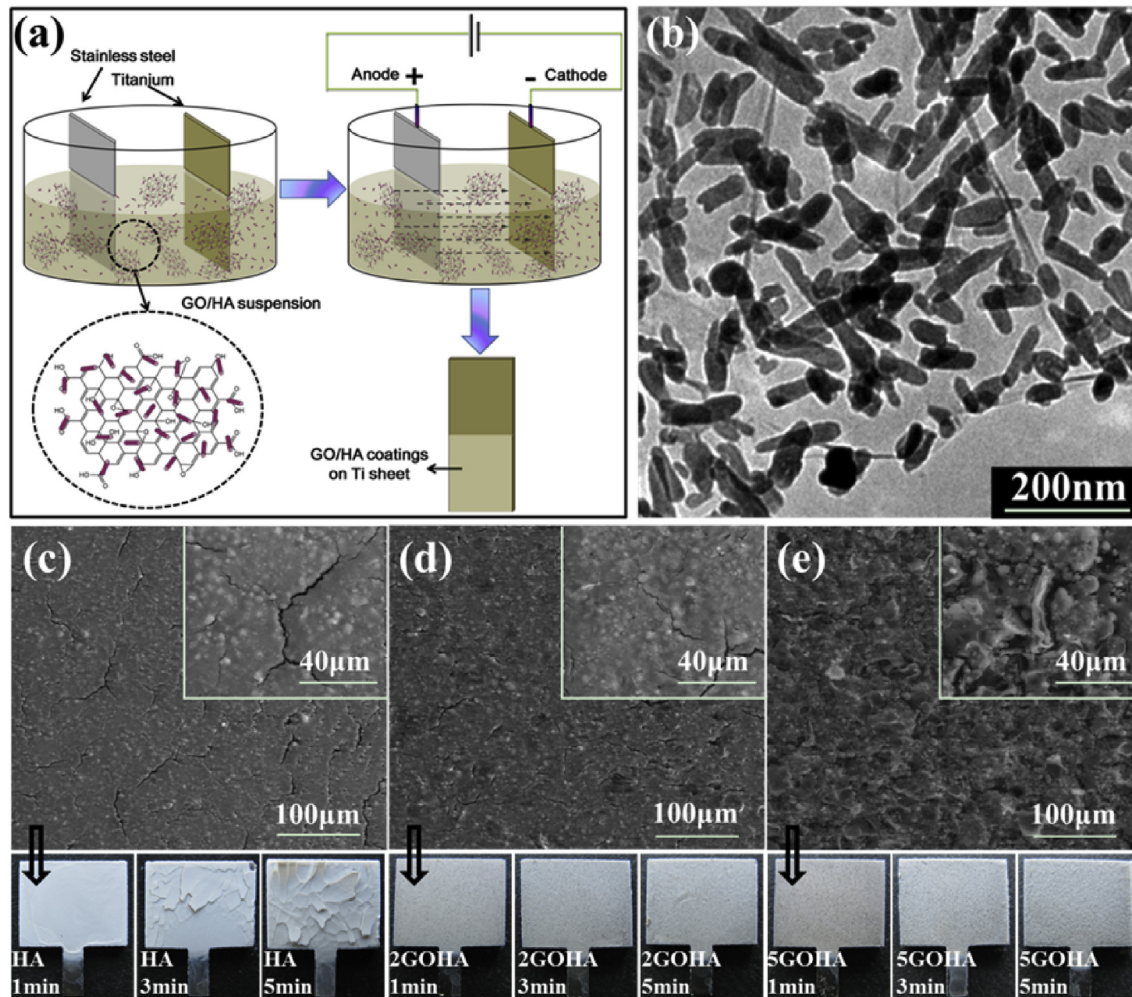
Electrochemical deposition of HA involves dissolving calcium and phosphate ions in a buffer solution with controlled pH values and temperature under varying electrical current [139]. When the voltage is applied,  $Ca^{2+}$  will migrate onto the cathode surface due to electrostatic attraction and react with the  $OH^-$  therein produced by the electrolysis of water, resulting in the *in situ* nucleation and growth of HA on the cathode surface [140]. Zeng et al. [106] fabricate GO/HA coatings on Ti by using this technique; GO was dispersed and mixed with electrolyte for deposition which consist of  $Ca(NO_3)_2$ ,  $NH_4H_2PO_4$ ,  $NaNO_3$  (to improve the ionic strength of the solution) and  $H_2O_2$  (to restrict the formation of hydrogen gas at cathode). The resulting pure HA coating exhibits a rough morphology with shell-like flakes and the GO/HA composite coating shows uniform and porous topography. The increase of GO contents in the electrolyte can improve the HA crystallinity and bonding strength of the coatings.

#### 2.3.3. Thermal spray

Thermal sprayed HA and HA-based composite coatings have been successfully used on commercially available Ti-based orthopedic implants, having the advantage of high deposition rate, good bonding strength and variable coating thickness [141]. This process involves heating the HA powders to melting state at high temperature, which may cause the decomposition of HA and exhibit detrimental effects on the coating biocompatibilities. Therefore, Liu et al. takes vacuum cold spraying as an alternative to prepare graphene/HA nanostructured coatings at room temperature [40]. The graphene/HA composite powder is prepared by wet chemical approach, and the sprayed coatings have tailorable thickness and display competitive adhesive strength and fracture toughness, with graphene evenly embedded in HA matrix [40]. The FESEM images of the coating were shown in Fig. 6 [40].

### 2.4. Graphene/HA composite scaffolds

Pure graphene 3D porous structures can be achieved by



**Fig. 5.** (a) Schematic illustration of the EPD process (b) The TEM images of the HA nanoparticles with 2 wt% GO sheets (c) The SEM images of composite coatings containing (c) 0 wt%, (d) 2 wt% GO and (e) 5 wt% GO sheets, and corresponding optical photos. These figures were adapted from Ref. [38].

chemical vapor deposition method with Ni foam as template [142] and hydrothermal approach [143] for tissue engineering applications. Moreover, graphene can be utilized as reinforcements for porous nanocomposites and this promising nanocomposite scaffolds can be produced by using lyophilisation or electrospinning.

#### 2.4.1. Lyophilisation

Lyophilisation is a frequently used technique to prepare porous scaffolds by freezing the solution and then reducing the surrounding pressure to enable the sublimation of frozen water. A scaffold with desirable mechanical and biological properties is obtained by lyophilizing the GO, HA and sodium alginate mixtures [76]. This novel nanocomposite scaffolds possess a porosity over 85% and average pore size larger than 150  $\mu\text{m}$ ; compared with HA 20 wt%- sodium alginate hybrid scaffolds, the addition of 1 wt% GO can improve their compressive strength and modulus by 23.2% and 28.3% respectively [76]. Nair et al. [68] incorporated the GO nanoflakes into gelatin-HA matrix by using freeze drying method and its morphology was shown in Fig. 7.

#### 2.4.2. Electrospinning

Electrospinning employs an electrical field produced under high voltage to force out the polymeric liquid from the spinneret, resulting in a polymeric fibrous and porous scaffolds on the

collectors [144]. Liang et al. prepared a composite nanofiber scaffold consisting of gelatin/chitosan/HA/GO by electrospinning [62] and the effects of the solution composition on fiber morphology were investigated; the addition of GO can obtain nanofibers with uniform and smooth microstructures and endows the fibrous scaffold with good antibacterial effect against both *Staphylococcus aureus* and *Escherichia coli*. Ma et al. [23] prepared a porous polylactic acid (PLA)/HA/GO scaffold using electrospinning method and SEM images of the composite were shown in Fig. 8.

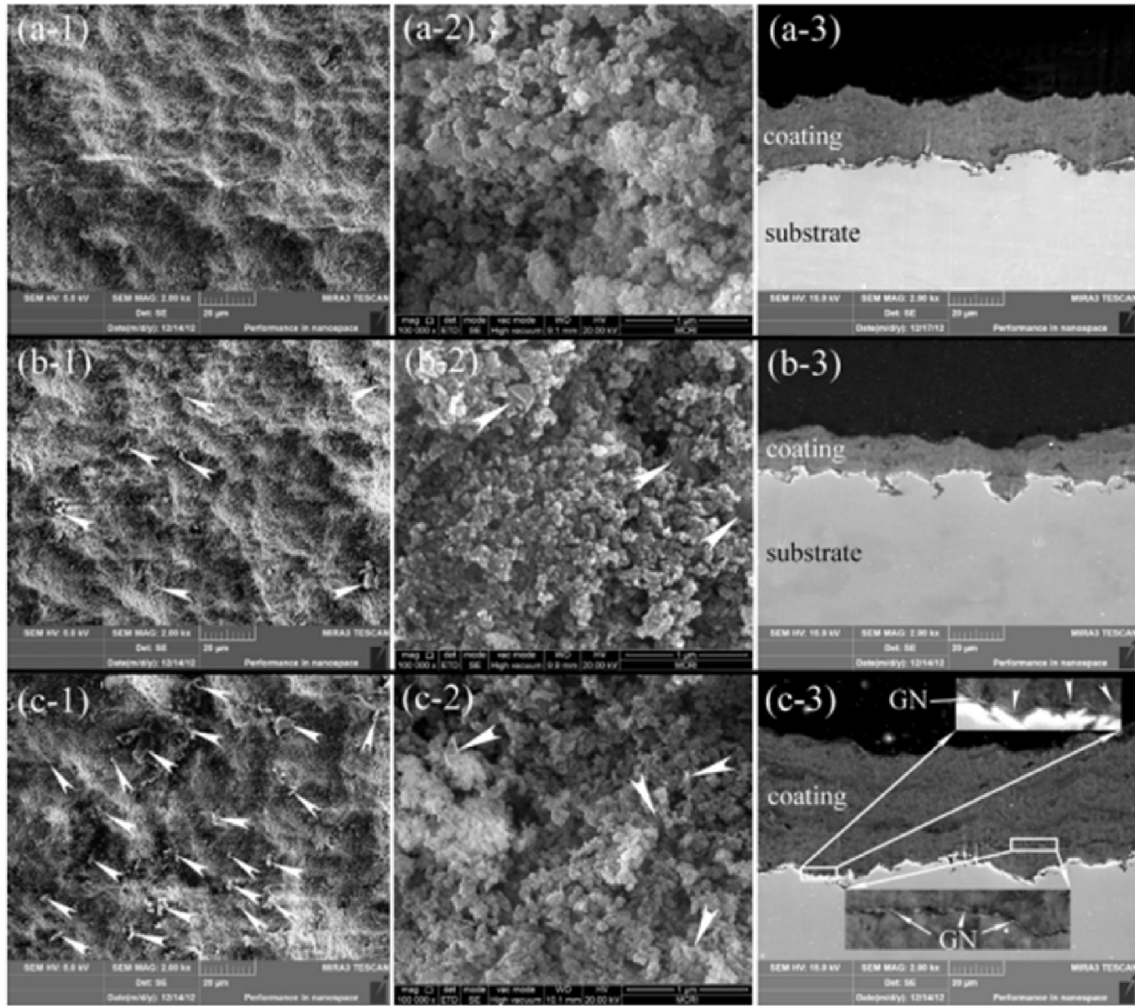
#### 2.4.3. 3D printing

Three dimensional printing is a superior additive manufacturing technique to print scaffold with customized shape, controlled chemistry and porosities and shows great potential for its application in bone tissue engineering [145]. Although bone has self-healing abilities, the large bone loss or damage cannot be healed completely and spontaneously. A scaffold or matrix materials should be incorporated to assist this healing process. Wu et al. [69] prepared GO surface modified  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) scaffolds by first using 3D printing method and then soaking the  $\beta$ -TCP scaffold into GO/water suspension as shown in Fig. 9.

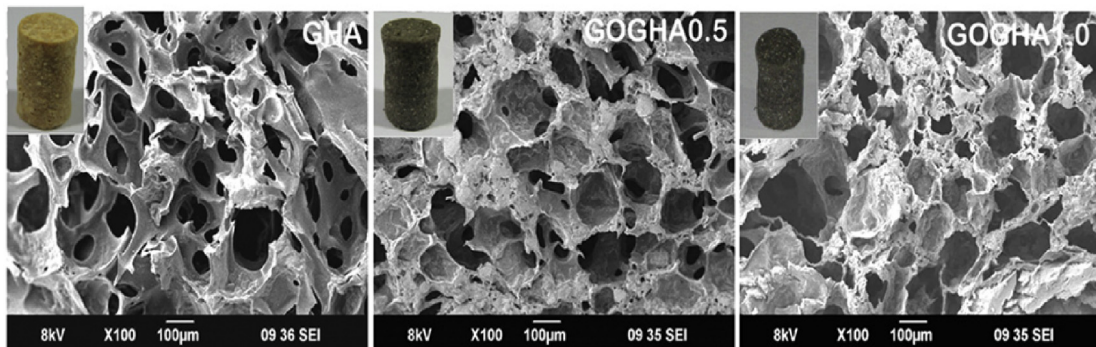
#### 2.4.4. Self-assembling

Self-assembly of GO provides a facile and efficient method to





**Fig. 6.** FESEM views of the as-deposited nanostructured coatings, (a) the pure HA coating, (b) the HA-0.1 wt% graphene coating, and (c) the HA-1.0 wt% graphene coating. –1: surface view, –2: magnified surface view, and –3: cross-sectional view. The white arrow head points to graphene located on the surfaces of the coatings, and magnified views of typical areas from the cross-section of the HA-graphene coating showing clearly the presence of GN in the coating and at the coating/substrate interface (c-3). Graphene-induced layered structure is clearly seen for the HA-graphene coatings.



**Fig. 7.** Scanning electron micrographs of the composite scaffolds. The inset demonstrates the photograph of the scaffolds. Note: GHA: gelatin-HA scaffold, GOGHA 0.5: gelatin-HA scaffold with 0.5 wt% GO, GOGHA1.0: gelatin-HA scaffold with 1 wt% GO [68].

produce graphene-based macrostructures. As shown in Fig. 10, GO and HA nanoparticles (nHA) were ultrasonically mixed in ice bath resulting in a homogeneous suspension, and then the mixture were heated at 200 °C for 3 h to induce self-assembly [122]. This technique reduced GO to rGO without using reductant and organic solvent, which could minimize cytotoxicity of the composite.

### 3. Mechanical properties of composites

#### 3.1. Mechanical properties of graphene and hydroxyapatite

As a biologically active calcium phosphate ceramic, its poor mechanical properties, such as low fracture toughness and tensile strength, limit its applications in major load-bearing scenario.

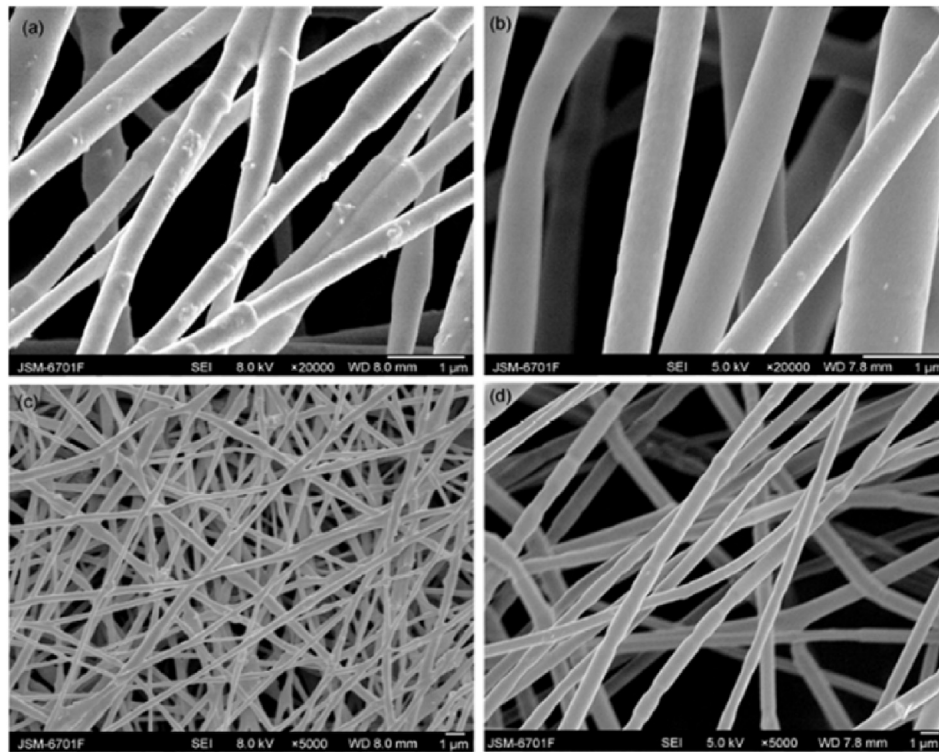


Fig. 8. SEM images of PLA/HA/GO nanofibers with high magnification (a) and low magnification (c), electrospun PLA with high magnification (b) and low magnification (d) [23].

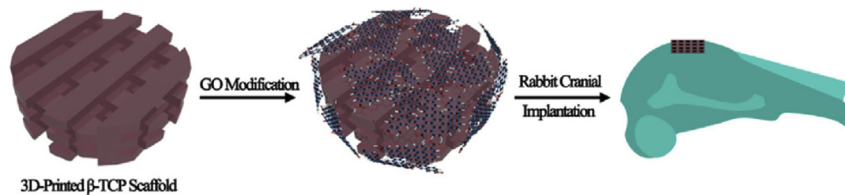


Fig. 9. Scheme illustration for GO modification of b-TCP bioceramics stimulates the in vivo osteogenesis [69].

Therefore, the main goal to incorporate graphene and its derivatives into HA is to improve the overall mechanical properties of the composite, in order to better fulfill its biological functions.

The atomically perfect monolayer graphene displayed a Young's modulus of 1.0 TPa and a fracture strength of 130 GPa, which was predicted to be stronger than any other materials [146]. The mechanical properties of graphene are influenced by the structural defects (monatomic vacancies and Stone-Wales dislocations) [147] and doping or functionalization defects [148]. As the oxygenated derivatives of graphene, graphene oxide contains reactive oxygenic groups, rendering it higher chemical activities for surface modification [149] but with lower effective Young's modulus ( $207.6 \pm 23.4$  GPa) [150]. GO could be reduced to reduced graphene oxide (rGO) with higher Young's modulus of 0.25 TPa [151]. Compared with pristine graphene, its oxygenated derivatives (GO and rGO) displayed higher stabilization behavior in aqueous media and could be attractive and promising nanoscale reinforcement fillers in biocomposites [38]. These nanofillers could enhance the interfacial bonding within the components, and facilitate stress transfer in the composites [152].

### 3.2. Mechanical properties of composites

Composites are expected to exhibit improved properties than

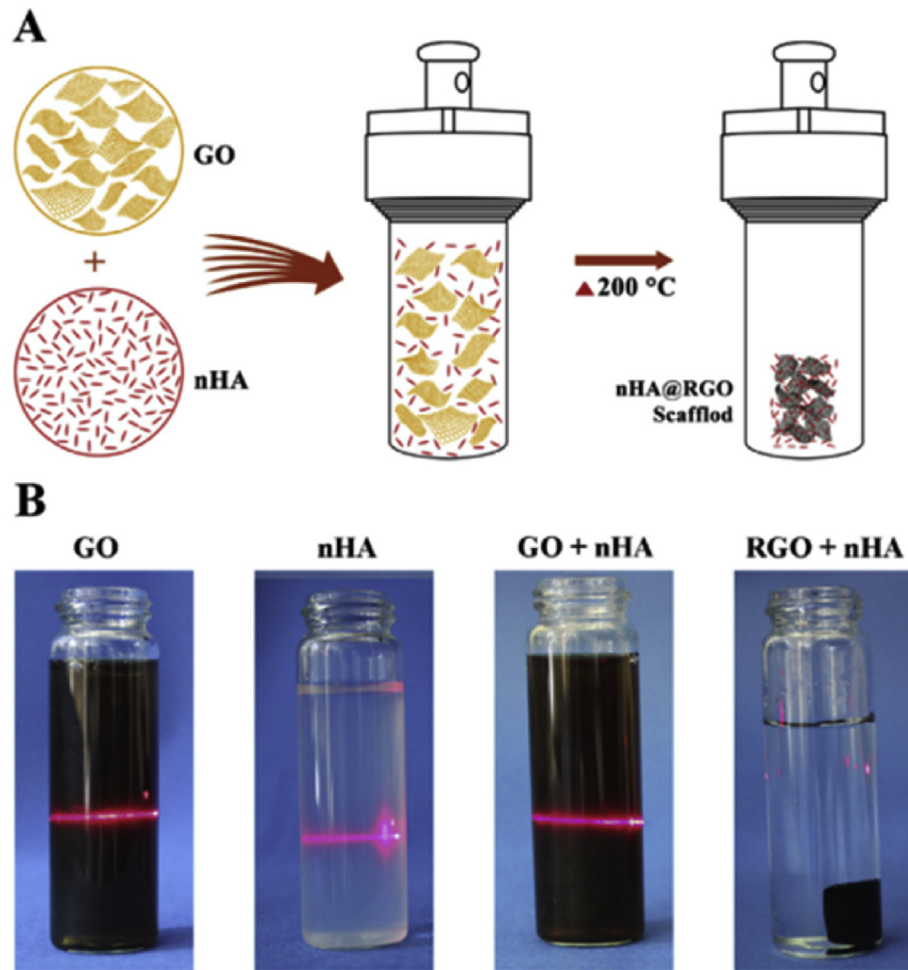
their individual components. The addition of GO could efficiently increase the adhesion strength of the HA coatings. The binding strength between the GO/HA coating and Ti substrate were evaluated according to ASTM F1044-99 in our previous research [30], and compared with pure HA coating, the adhesion strength of 5 wt %GO/HA coating increased from  $1.55 \pm 0.39$  MPa to  $3.3 \pm 0.25$  MPa.

The rGO/HA composites were prepared by hydrothermal approach and consolidated by hot isostatic pressing technique; and then their mechanical properties were assessed using the indentation method as shown in Fig. 11 [28]. According to the investigation of S. Baradaran et al., even low addition of rGO has a great effect on the bulk mechanical properties, and the composite containing 1.5 wt% rGO displayed a maximum fracture toughness and elastic modulus, around 86% and 40% higher than that of the pure HA [28].

### 3.3. Toughening mechanism

The mechanical efficiency of the reinforcement fillers in the composites were mainly determined by several factors: (1) the intrinsic mechanical properties of the fillers, (2) the inherent mechanical behavior of the matrix, (3) the fillers volume fraction, (4) the preparation method of the composites, (5) the interfacial bonding strength between the filler and matrix, and (6) the





**Fig. 10.** (A) Schematic showing that the self-assembly of RGO and nHA to form a porous RGO scaffold for cranial bone defect reconstruction; (B) Tyndall effect of before and after reaction [122].

distribution level of the fillers within the matrix [44,153].

The toughening mechanism for the graphene-based HA composites, according to previous investigations [25,31,32,44], are crack branching, crack bridging, pull out, crack deflection and other reinforcing mechanisms. To be specific:

- (1) The addition of the 2D fillers can result in the formation of HA with smaller sizes [32,154]; and the GO/rGO may be functionalized as nucleus for the crystallization of HA crystals [30], which in turn may restrain the growth of HA nanoparticles. It is reported that the fine grain strengthening mechanism could be a possible cause for the improved mechanical properties of the HA composites [32]. This inhibitory effect on grain growth was well studied by Liu et al. [90] and illustrated in Fig. 12.
- (2) Moreover, the calculated pullout energy of graphene from HA is around  $3\text{--}40\text{ J m}^{-2}$ , which is much higher than the fracture energy ( $1\text{ J m}^{-2}$ ) of bulk HA [32]. Therefore, the indentation-induced cracks, as shown in Fig. 11, preferably propagate within the HA matrix, instead of along the HA-graphene interface. The stress transfer characteristics in graphene-based nanocomposites were evaluated from a computational model using a multi-scale finite element approach [102].

- (3) Graphene-based nanosheets have high specific surface area enabling increased contact area with the matrix [25]. And due to its high Young's modulus and flexibility, graphene may be located around the grain boundaries and aligned with the grain shape [31], which is expected to enhance the mechanical interlocking and adhesion strength within the matrix [30]. The graphene-based 2D reinforcement fillers can inhibit the propagation of the crack. Liu et al. [40] prepared graphene/HA coatings by vacuum cold spraying and the authors incubated the samples in culture media for one month without cells, and the coating adhesion values showed no remarkable changes.

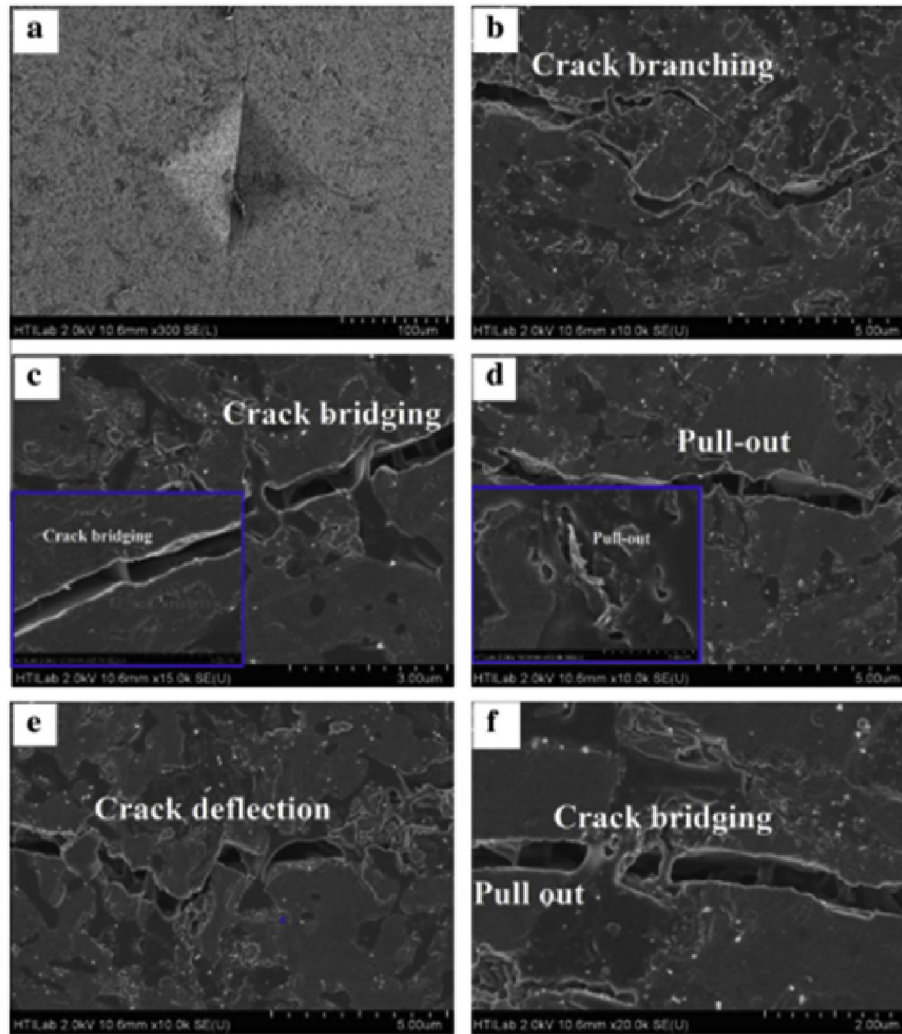
## 4. Biocompatibility of the composites

### 4.1. *In vitro* biocompatibility

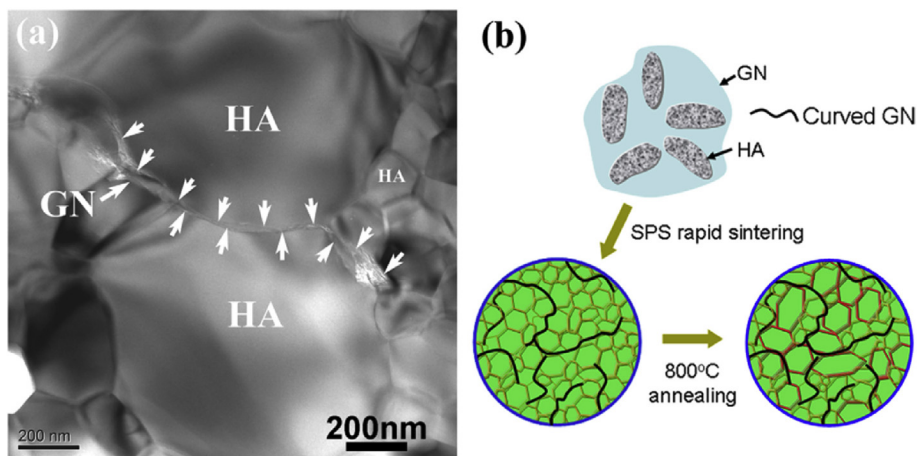
From a mechanical point of view, this graphene-based HA nanostructural composites have proven to be promising candidates for hard tissue repairing or regeneration. Furthermore, these materials should be biocompatible with high stability in biological environment. Their toxicity was well analyzed and studied.

#### 4.1.1. Cytocompatibility

Usually the graphene-based HA composites were co-cultured



**Fig. 11.** Characteristic toughening mechanisms at a striation line in the composites with 1.5 wt% rGO: Vicker's indentation craters (a) and radial cracks: crack branching (b), crack bridging (c and f), pull out (d and f), crack deflection (e) [44].



**Fig. 12.** Restrained HA grain growth by graphene and abnormal growth of the grain along the direction parallel to graphene, (a) TEM image of the HA-graphene composites showing that graphene is predominantly located at the HA grain boundaries, forming a serial wall zones isolating individual HA grains, and abnormal HA grain growth is seen along the direction parallel to graphene, and (b) schematic depiction of the composites illustrating evolvement of the HA grains during the SPS processing and following heat treatment.

with (1) osteoblast-related cells: mouse pre-osteoblast cell line (MC3T3-E1) [17–19,23,25,35,37,46,71,74], human fetal osteoblastic

cell line (hFOB 1.19 cells) [39,40,44,56], human osteosarcoma cell line (MG63 cells) [30,34,38,155]; (2) fibroblast-related cells: mouse

embryonic fibroblast cell line (NIH 3T3 cells) [34], mouse fibroblast cell line (L929 cells) [24,30,33,38], (3) stem cells: mesenchymal stem cells (MSC) [28] and (4) other cells: PBMC (human peripheral blood mononuclear cells) [53,67].

Fan et al. [46] seeded MC3T3-E1 cells onto graphene/HA composites with various HA contents and the cells displayed flatter morphology than on the GO and HA coating, and the composite containing 40 wt% showed higher bone cellular activities. In order to further increase the biocompatibility of the graphene/HA composites, bioactive polymers were added into the system, such as carrageenan [35], gelatin [37] and polydopamine [74]. Combined with the graphene-based fillers, these polymers could facilitate the HA mineralization process which promoted the osteogenic differentiation of MC3T3-E1 cells. Lee et al. [63] cultured the MC3T3-E1 cells ( $2 \times 10^5$  cells/mL) with colloidal dispersion of HA particles, rGO nanosheets and rGO/HA nanocomposites (10  $\mu\text{g/mL}$ ) for 1–21 days and the cells were analyzed by Alizarin red staining (ARS). The results were shown in Fig. 13.

Oyefusi [39] investigated the hFOB 1.19 cell proliferation and differentiation by using total protein assays and Western blot analysis of osteocalcin expression with promising results. Liu et al. [40] cultured the osteoblast cells on the composite coatings showing higher proliferation rate and better stretching behavior on the HA-based coatings than on bare Ti. The random curly incorporated graphene sheets possessed more active sites for cell binding and could adsorb the key serum proteins (fibronectin) to further enhance the cell attachment [40].

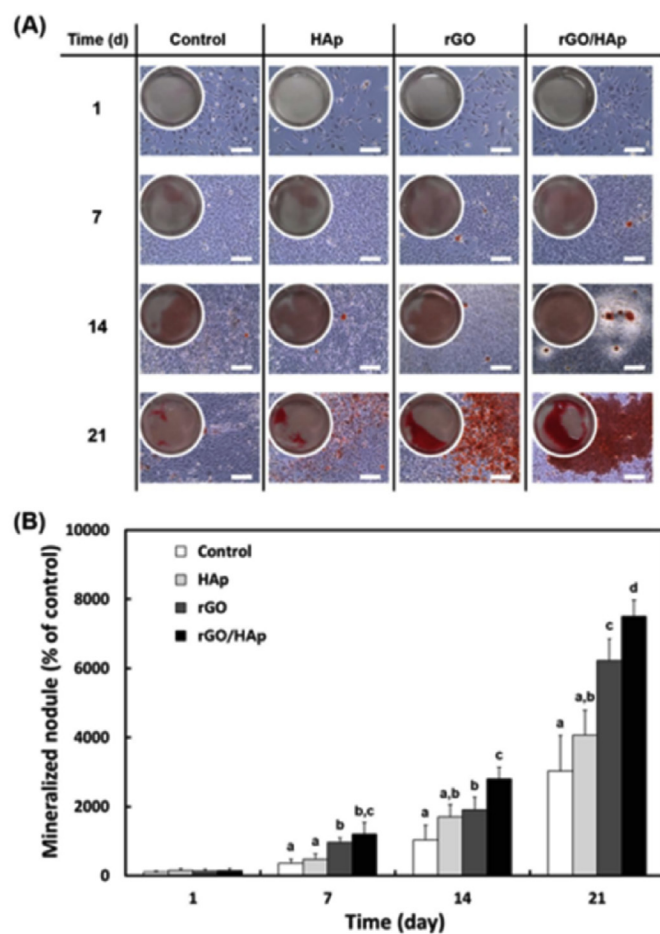
The graphene/HA composites also showed good cytocompatibility to MG63 cells. The HA coated GO exhibited comparable biocompatibility to HA minerals [155]. However, high GO contents in the composites might inhibit cell viabilities [38]. Compared with the GO/HA composites, the addition of chitosan could increase its bone-forming abilities [30]. Ramani et al. [34] prepared bacterial cellulose/GO/HA composites with high osteoinductive and the MG63 cells cultured with 50  $\mu\text{g/mL}$  composites had higher ALP activities.

Liu et al. [24] evaluated the cytotoxicity of rGO/HA on L929 cells using MTT assay and the cell viability was more than 95% in comparison with the control; moreover, the concentration of this composite in the culture media (0, 1, 5, 10, and 20  $\mu\text{g/mL}$ ) had no significant influences on the mitochondrial activities within the L929 cells. Wang et al. [33] prepared a GO/fibrinogen nanofiber scaffold by using layer-by-layer method and then incubated it in SBF for biomineralization resulting in 3D GO/HA scaffolds, which had no obvious inhibitory effects on the *in vitro* cell proliferation of L929 cells. In our previous study [30,38], the *in vitro* cytotoxicity of the GO/HA and chitosan modified GO/HA did not exhibit obvious concentration-dependent characteristics but showed a clear positive time dependence [30]; and the cells displayed a healthy round shape with extended pseudopodium [38].

Zanin et al. [28] prepared globular nano-HA onto rGO by electrodeposition and evaluated the composite's biocompatibility by culturing with MSC. The cells adhered well on the composites with a flat roughly circular morphology, presenting active formation of membrane projections. PBMC consists of lymphocytes and monocytes, as the main representatives of human immune cells [53]. Compared with the control group, the cells viability rate on the composites showed mild decrease and the composite could be considered non-toxic.

#### 4.1.2. Hemocompatibility

Nair et al. [68] incorporated the GO nanoflakes into gelatin-HA matrix by using a freeze drying method. None of the prepared scaffolds showed hemolysis with comparable activity to the saline control groups [68]. In our previous study [88], silk fibroin/GO/HA



**Fig. 13.** The ARS stain and its corresponding extract in MC3T3-E1 cells incubated with a colloidal dispersion of HAp particles, rGO nanosheets or rGO/HA nanocomposites in basal media. (A) Increased calcium deposits by rGO/HA nanocomposites were not related to the cell number (scale bars = 200  $\mu\text{m}$ ). There was a notable formation of calcium deposits by rGO/HA nanocomposites from 14 d. (B) The dissolved ARS extracted from the staining plates confirmed that the rGO/HA nanocomposites significantly ( $p < .05$ ) increased extracellular calcium deposition in the cells [63].

coatings were electrophoretic-deposited on Ti substrates and the hemocompatibility of samples were evaluated according to ASTM F756-08 standard [156]. All the samples exhibited good hemocompatibility with hemolysis rate lower than 5%, and platelet adhesion tests showed that only few platelets were observed on the composite coatings at the inactivated stage with round shape (Fig. 14). The GO and graphene nanosheets showed a dose-dependent hemolytic activity on red blood cells [157] and covering GO sheets with HA could alleviate their hemolytic activity.

#### 4.1.3. Mineralization abilities

Simulated body fluid (SBF) contains ion concentrations almost equal to those of human blood plasma. Usually the bone-forming or bone-bonding abilities of a material could be evaluated by immersing it into SBF to examine apatite formation abilities on its surface, which is a useful method to predict the *in vivo* osteogenic activity of a material [158]. However, some researchers suggested that this method needs further elaboration [159].

The addition of graphene-based nanofillers into HA matrix could accelerate the formation of apatite on its surface [25,42,46,53,78]. Zhang et al. [25] prepared graphene/HA composites using spark plasma sintering technique. The samples were immersed in SBF for 7 days, and, compared with the HA, the bone-



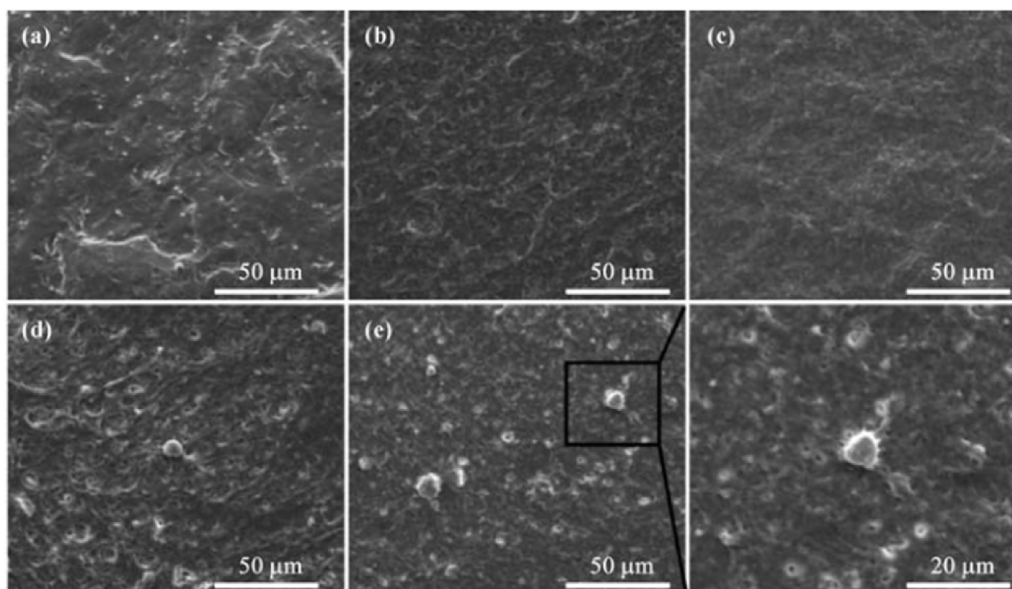


Fig. 14. SEM images of the adhered platelets on pristine (a) Ti, (b) SF, (c) SF/GO, (d) SF/HA and (e) SF/GO/HA coatings [88].

like apatite layer formed on 1.0 wt% graphene/HA was much thicker than that on the pure HA substrate, probably suggesting a higher osteogenic activity of the graphene-reinforced HA [25]. The proposed mineralization process were depicted in Fig. 15. Compared with pure HA, the graphene/HA composites exhibited lower stability. The incorporation of graphene into HA could result in smaller grain size and more specific area, leading to fast dissolution of calcium and higher negative surface charge on the composites (Fig. 15 a). Therefore, more calcium could be attracted on its surface (Fig. 15 b) and the Ca-rich layer could generate thicker bone-like layer (Fig. 15 c) [25,53]. In order to further increase the bone-forming ability of the composites, the nanosheets could be coated by bioactive polymers such as polyethylene glycol [42] and self-assembled peptide nanofibers [78].

#### 4.2. In vivo biocompatibility

Bioactivity of the graphene-based HA composites in the context of osteogenesis by using *in vitro* cell models have been extensively

investigated. However, there were few *in vivo* animal studies using these novel material.

Lee et al. [63] prepared the HA grafts and the rGO/HA grafts. The grafts were filled into bone defects (6 mm in diameter and 2.5 mm in depth) that were trephined in the parietal bone of 12–13 week-old male New Zealand rabbits [63]. Four weeks after the surgery, the non-treated control defects were filled with thin and loose connective tissues without many new bones (Fig. 16A(a-c)), and dense connective tissue and small particles were observed in the one with HA grafts (Fig. 16 A(d-f)). For the groups implanted by rGO/HA grafts (Fig. 16A(g-i)), several newly formed bone were detected, indicating the accelerated bone remodeling process. From the Masson's trichrome staining analysis, the amount of the newly formed bone in the rGO/HA treated defects (Fig. 16 B(c,d)) were substantially higher than that in the HA groups (Fig. 16 B(a,b)). The histometric evaluations (Fig. 16 C) showed that the rGO/HA grafts displayed significantly greater new bone density than the control/HA groups.

Wu et al. [69] prepared GO modified  $\beta$ -tricalcium phosphate ( $\beta$ -

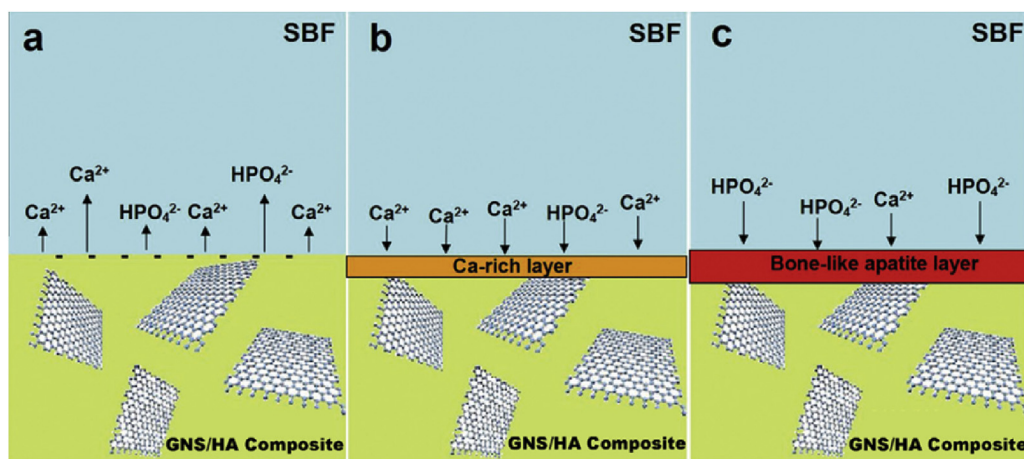
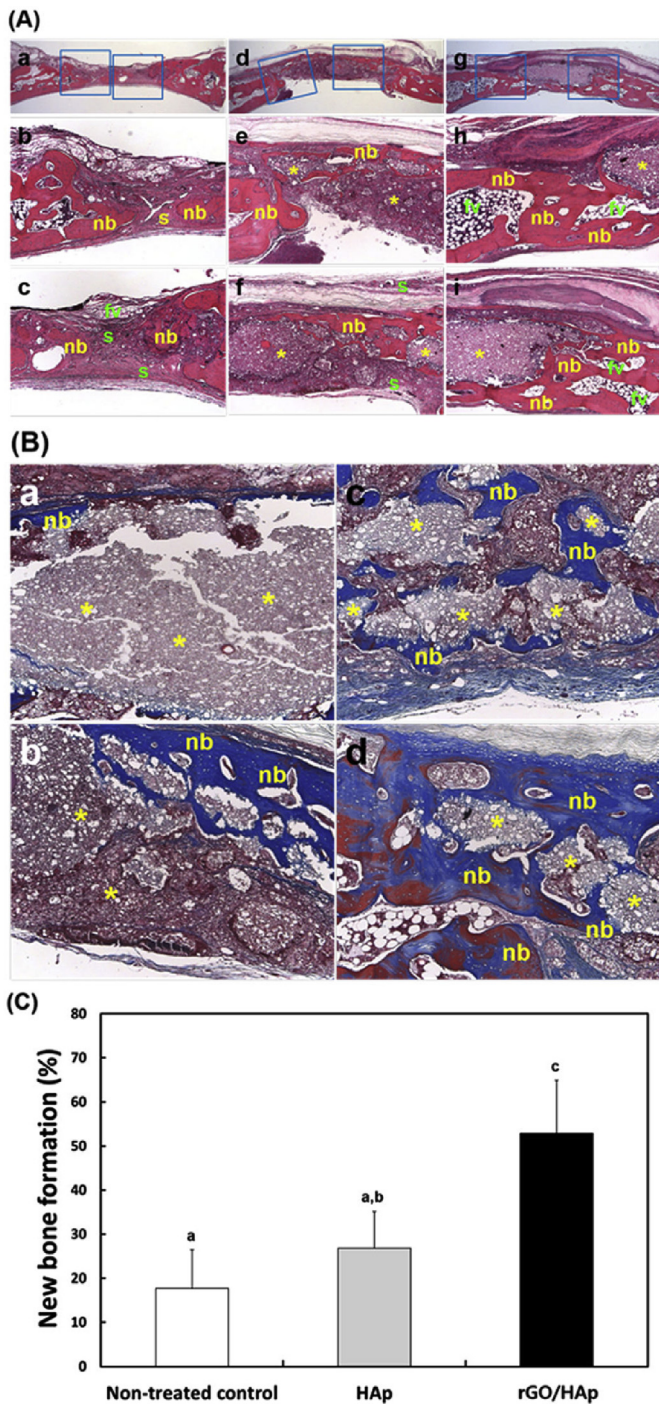


Fig. 15. Schematic illustration of the mineralization process for a GNS/HA composite immersed in SBF. (a) Dissolution controlled stage, in which calcium ions dissolution is predominant, leading to negatively charged surface. (b) Precipitation controlled stage, in which negatively charged surface together with more nucleation sites attract calcium ions from SBF to form the Ca-rich layer. (c) Formation of bone-like apatite, in which Ca-rich layer attracts phosphate ions and form bone-like apatite [25].



**Fig. 16.** Histological observations. (A) Images from HE staining: non-treated control (a–c), the defects treated with the HAp grafts (d–f), the defect treated with rGO/HAp grafts (g–i) Original magnifications:  $\times 12.5$  in (a,d,g) and  $\times 50$  in the others. (B) Images from MT staining. (a,b) HAp grafts and (c,d) rGO/HAp grafts. Original magnifications:  $\times 100$ . Symbols: nb, new bone; s, soft tissue; fv, fibrovascular tissue; \*, graft materials. (C) New bone formation (%) [63].

TCP) scaffolds by first using 3D printing and then soaking the  $\beta$ -TCP into GO/water suspension. The *in vivo* bone formation of the scaffold was evaluated by implanting it into critical-sized calvarial defects in New Zealand white male rabbits. Compared with  $\beta$ -TCP scaffolds, the GO modified scaffolds had greater bone formation abilities in the defects both at 4 and 8 weeks post implantation. And the new bone was visible in the periphery and center of the defect

in the GO- $\beta$ -TCP group, which was both quantitatively and qualitatively better than that in the control group as it is shown in Fig. 17.

Both the *in vitro* and *in vivo* biocompatible analysis suggested that the graphene-based HA composites showed great potential applications for bone reconstruction, due to its beneficial effects on the adhesion, proliferation and differentiation of osteoblast-related cells, fibroblast-related cells or stem cells. Graphene and its derivatives displayed strong non-covalent binding abilities on osteogenic inducers (dexamethasone and  $\beta$ -glycerolphosphate) [160] which accelerated the osteogenic differentiation of stem cells.

#### 4.3. Antibacterial property

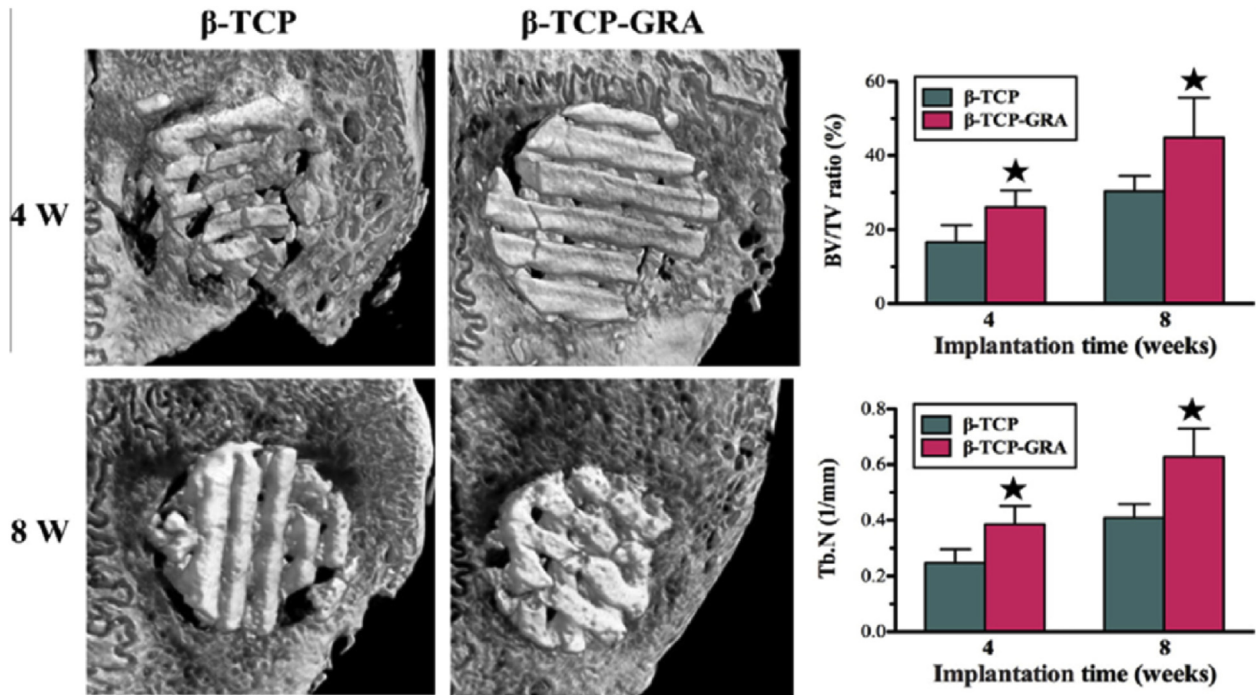
The infections of the implantation site after surgery [67] and biomedical devices associated infections (especially with the formation of biofilm) [100,161] are the major cause for delayed healing, implant failure and repeated surgeries [161]. Therefore, it is necessary to develop novel composite materials with combined bioactivity and antibacterial properties.

In addition to the bone-forming ability, HA shows favorable affinity for bacterial adhesion [100]. When HA was exploited as coating materials on Ti implants, the increased HA contents therein resulted in enhanced attachment of the bacteria, which could deteriorate its osteointegration property and further weaken the biological fixation ability of HA-coated implants in hard tissues [162]. Accordingly, it is imperative to endow HA with antibacterial or bacteriostatic effects by preparing HA composites. Graphene-based nanomaterials exhibit excellent cytotoxicity to bacteria [163]. Liu et al. [164] investigated the antibacterial activity of four types of graphene-based materials (graphite, graphite oxide, GO and rGO) toward an *Escherichia coli* and the GO dispersion displayed the highest antibacterial activity. The membrane stress induced by the nanosheets warping [164], the cell membrane damage [165] caused by direct contact with sharp edges of the nanosheets and the oxidative stress generated by the reactive oxygen species (ROS) production [164] were the major mechanisms in the bacterial inactivation.

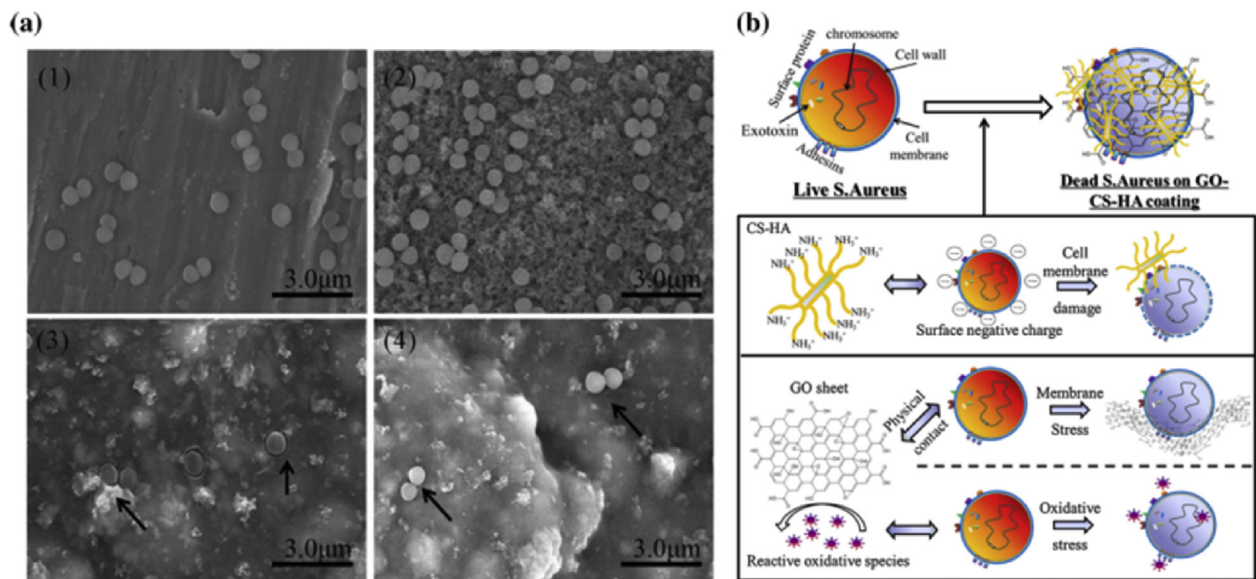
Janković et al. [53] investigated the antibacterial efficiency of the electrophoretic graphene/HA coatings against the Gram-positive pathogenic bacteria strain *Staphylococcus aureus* and the Gram-negative bacteria strain *Escherichia coli*. However no antibacterial effects were observed in that study [53] which might be ascribed to the low contents of the incorporated graphene. As an alternative method, Ag was introduced into the coatings, and the graphene/Ag/HA coatings showed strong antibacterial activity only after 3 h coculturing with the *S. aureus* and *E. coli*, inhibiting harmful biofilm formation [67]. In our previous research GO reinforced chitosan/HA coatings were deposited onto Ti and the antibacterial adhesion assay indicated that the amount of the adherent bacterial cells decreased greatly on the composite coatings compared with pure HA coatings [100]. As shown in Fig. 18 (a), compare with Ti and HA coating, the number of the adherent bacterial cells is significantly decreased on the chitosan/HA and GO/chitosan/HA coatings, with the potential antibacterial mechanism displayed in Fig. 18 (b) [100].

The novel graphene-based HA composites were competitive candidates for hard tissue repairing or regeneration with increased osteogenic activity, well hemocompatibility, and promising antibacterial properties. However, in some cases the resulting composites exhibited less bioactivity [24,38,53]. This discrepancy may originated from the preparation techniques of graphene, the size and distribution of this 2D nanoscale filler. In the scenario of graphene oxide, the types of oxygen-containing functional groups and the oxygen content could also affect the bioactivity of the composites. Therefore, more researches should be conducted to shed light on this issues.





**Fig. 17.** Micro-CT analysis of in vivo bone formation ability for  $\beta$ -TCP and  $\beta$ -TCP-GRA scaffolds after being implanted in the cranial bone defects of rabbits for 4 and 8 weeks. \*Significant difference between the  $\beta$ -TCP and  $\beta$ -TCP-GRA groups ( $p < .05$ ). Note:  $\beta$ -TCP-GRA represents GO-modified  $\beta$ -tricalcium phosphate and  $\beta$ -TCP represents  $\beta$ -tricalcium phosphate [69].



**Fig. 18.** SEM images (a) of the *S. aureus* after incubation 12 h with the pristine Ti (1) and different coating interfaces: HA (2), chitosan/HA (3), GO/chitosan/HA (4). The black arrows in (3), (4) point to the adherent *S. aureus*. Scale bar 3  $\mu$ m. Schematic illustration (b) of the speculated antibacterial adhesion mechanism of the GO/chitosan/HA nanocomposites.

## 5. Conclusions

This review of literature presented various preparation techniques, mechanical behaviors and toughen mechanism, *in vitro/in vivo* biocompatible analysis, antibacterial properties of the graphene-based HA composites for orthopedic applications. By combining the superior mechanical properties of graphene (and its derivatives) and the high bioactivities of HA, the graphene-based HA composites show great potentials in hard tissue repair and

regeneration. The major problem in production of this novel composite is the difficulty in homogeneous distribution of the 2D reinforcement fillers within the matrix. Evaluation of the composites mechanical properties has been the focus of the research. Biocompatibility of graphene-based HA composites has been studied mostly in terms of *in vitro* cytocompatibility, and further *in vivo* analysis should be conducted before being considered for clinical applications.



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