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Graphene Family-Calcium Phosphates for Bone Engineering and their Biological Properties

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Abstract. Graphene family (GF)-Calcium phosphate (CaP) composites, holds great potential as components of bone regeneration materials. GF nanomaterials can be modified physically as well as chemically, which are to be biomimetic, mechanically to be hard due to their capability to support exceptional mechanical, thermal and electronic properties. These biocompatible GF composites coated on the calcium phosphate can enhance and tolerate stem cell growth and differentiation, proliferation into various lineages or interact with bioorganisms. The development in CF-CaP materials and their physicochemical/biointeractions and strategies towards osteoblasts is a great concern to promote faster healing and reconstructions of large bone imperfections or defects. In this review, we explain the influence of the CF-CaP and summarize recent developments on designing CF-CaP architectures as multifunctional bone regeneration platforms. We have also explored the typical biological applications concerning these CF-CaP based bioactive nanocomposites. Furthermore, the future viewpoints and evolving challenges will also be emphasized. Due to the shortage of full-length reviews in this emerging research field, this review would be caught great attention and encourage various new chances across a wider range of disciplines.

Keywords: Graphene-Calcium phosphate; nanocomposites; osteogenic differentiation; mechanical properties; bone tissue engineering

1. Introduction

Bone tissue regeneration is of high attention to upgrade faster healing and restoration of large bone defects formed by skeletal abnormalities, tumor resection, fractures and infection[1]. This review emphasis on graphene family (GF)-calcium phosphate (CaP) (graphene family i.e Graphene (G), Graphene oxide (GO), reduced graphene oxide rGO) based bone substitute materials which can be used for bone repair, bone replacement, regeneration or augmentation[2-8]. GF-CaP biomaterials had paid much attention due to its bioactivity[9,10]. Thus, this review will also contain the effect of the surface structure of calcium phosphate as well as its physico-chemical properties after the addition of GF nanomaterials[11,12].The development of this field needs the utilization of substrates that aid cell connection and differentiation[13-15]. Many diversity materials can stimulate, initiate and tolerate the series of complex actions that can make cell differentiation as well as osteogenesis. In general, collagen can exhibit proper surface chemistry for cell differentiation and cell growth but holds poor mechanical properties and is disposed to immune response.[13,16,17] Hydrogels, which are with tunable physicochemical properties, may positively direct stem cell purpose. Yet, their frontiers may include deficiency of cell-specific bioactivities and it is puzzling to generate bulky structures due to the requirement of a highly bridged network that can affect cell behavior.[18-20] So, materials with significant characteristics needed to survive cell growth and encourage differentiation to hold a boundless potential for stem cell research.

Graphene and its derived products GO and rGO have acknowledged increasing attention for biomedical applications as they can show remarkable properties such as high surface area, high mechanical strength, electrical strength, and an ease of chemical modification.[13,21-25] Graphene is specially framed with sp^2-bonded carbon atoms[26]. In addition, Graphene is the thinnest and most durable monolayer and lightest material and it can exist freely. Individual graphene nano sheets were initially separated from graphite when Scotch tape had been used to peel for preparing graphene include growth from a raw carbon source, cutting open carbon nanotubes, sonication, and reducing carbon dioxide or graphite oxide[27,28].In addition, GO is an outstanding hydrophilic material by introducing oxygen-containing functions onto the graphene surface, such as hydroxyl, carbonyl, carbonyl, and carboxyl. Owing to its good dispersity in water, high featured ratio and pleased mechanical properties, GO has become a best competitive material to reinforce cementitious materials.[29,30] Lu et al[31] reported that 0.05 wt. % GO lead to 11.1% and 16.2% rise in flexural strength and compressive strength of the cement paste. Duan et al[32] demonstrated that 0.03 wt. % GO sheets enhanced the tensile strength and compressive strength of the Portland cement composite by more than 40% as a result of the reduction of the pore structure. Nevertheless, fewer studies examined the outcome of GO on the properties of the magnesium potassium phosphate cement (MKPC) paste[33]. rGO can exhibit powerful factors in helping the spontaneous osteogenic differentiation of osteoprogenitor cells as well as visualizes that the rGO would be the potential candidate for scaffolds in tissue engineering, stem cell (SC) differentiation and constituents of implantable devices, due to its biocompatible and bioactive properties[34]. GF-CaP based nanomaterials showed great interest on osteoinductivity with the Western blotting, immunocytochemistry, and the Alizarin red staining assay with any increased rGO in GF-CaP materials, which is due to the distinct surface properties and chemical properties of GF-CaP. Many studies have been enthusiastic to the behavior and toxicology of calcium phosphate coated graphene nanomaterials both in vitro and in vivo.[35-37] GF-CaP can enhance the adsorption of extracellular biomolecules, specifically proteins, consequently accelerating the extracellular matrix (ECM). This finally helps cell colonization by providing an extra beneficial microenvironment for cell adhesion and growth. Inspite of there are already a huge number of reports that have been devoted to the investigationof GF-CaP-coated bioactive materials, we noticed that acomprehensive review in this specific field is still lacking. Therefore, we will review the recent developmentsand perspectives for the application of physico-chemical interactive or biointeractive GF-CaPcoatings in biological zones. In this perspective, we will explain bone structure and properties, chemistry and bioorganisms interactionsbetween GF-CaPs. Finally, we will discuss the typical biological applications onthese emerging biomedical applications of GF-CaP coatings.

2. Structural and Chemical Properties of Bone

Bone is a basic unit of the human skeletal structure and functionally graded material with an inner cancellous as well as outer cortical bone.Different coatingsystems impact the improvement of macroscopically varied bone structures[25,36-38] (Fig.1)].Bone is a remarkable tissue, a complex composite of biomineral polymer. The

biopolymer contains proteins, generally collagen [type 1] and significant non-collagenous proteins like proteoglycans, osteogenic factors (e.g., bone morphogenetic proteins) and minor amounts of lipids[39].
In addition, bone in any healthy living organism, adapt to the loads under which it is located[42,43]. If loading on the bone increased, it will adjust itselfover time to become stronger by first altering the interior architecture of the trabeculae, later thickening the external cortical portion of the bone. Conversely, if the loading on the bone decreases, then it will become lesser dense due to the lack of the stimulus essential for continued remodeling, where this process regarded as osteopenia. It is also noteworthy to understand the structural interactions and relationship between the several levels of hierarchical structural organization to know the purpose of hydroxy apatite: (1) the microstructure and sub-microstructure:Haversian canal, osteons, lamellae; (2) themacrostructure: cancellous versus cortical bone; (3) The nanostructure: fibrillar collagen; (4) the sub-nanostructure: molecular constituents of the mineral, collagen, and non-collagenous organic proteins
The microstructure of bone contains mineralized collagen fibers to make it into planar arrangements called lamellae[44,45] (Fig.1a& Fig.1b). As shown in Fig.1a, in some cortical bone the lamellae wrap in concentric coatings around a central canal, to builda osteon (i.e a Harversian system). The osteons usually appearas rolls, and they are coarsely comparable to the long axis of the bone[45].
On the macroscopic stage, bones have varied forms based on their respective purpose[46].See Fig.1c Cortical bone produces the outer shell of bones. It is a properly dense bone, with the porosity[44 – 46]in the order of 6%. The interrelating framework of trabeculae is in a several numbers of combinations, in which all are following basic cellular structures: rod-rod, rod-plate, or plate-plate[45-49]. In case of the trabecular bone, it is having pores in the order of 80% [50] and the typical thickness is about 50–300 μm which depends on the load distribution in the bone with respect to the orientation[51]. When directing in vivo study of osseointegration of coated and uncoated implants, oneshould consider into an account the reactivity of bone surrounding the implant. Namely, at the diaphysis (Fig.1c), native bone is in neighboring contact with an implant. The meta physic (Fig.1c), on the other hand, it consists cancellous bone which is more reactive & hence it provides faster facture healing[52].
In the lamellae's nanostructure, there is a mineralized collagen fibril, which is about 100 nm in diameter. The collagen in the fibrils also limits the probable primary growth of the crystals, driving them to be discrete and discontinuous. Crystals gained at regular intervals beside the fibrils, with a roughly repeated distance of 67 nm [53, 54], which agree to the distance by which neighboring collagen molecules are staggered. It is thus significant to notice that the arrangement of the lamellae and collagen fibres up to the nanoscale increase the isotropic assets found in bone hinder, the crack proliferation, and enhances robustness[53].
The generation of the apatite in the extracellular region of the collagen is called biomineralization. The nucleation procedure in the bones is allied with interaction among anionic proteins and type I collagen fibrils which may provide the stereochemical alignment of negatively charged functions that is adequate for hydroxyapatite nucleation. When the bone matrix is generated, a representative time course of nearly 2 weeks will take place before the matrix initiates to mineralize speedily. This mode is called primary mineralization, and within a few days, the matrix can be mineralized up to 70%. The residual 30% of enhancement in mineral content lasts numerous years, and is called secondary mineralization[55]. Epitaxial concerns have been found to be of main significance i.e biomineralization, in apprehension the generation of teeth and bones, as well as in pathological ways eg. The progress of urinary calculi [45-54,56].
Regarding the structure of bone mineral crystals, the major studies revealed them as plate-like in structural shape [The thickness of the platelets is between 1.5 to 9 nm, while the length is about 15-200 nm, and the width is about 10-120 nm [57-65]. The circumstances in the human being body apparently permit limited growth of hydroxyapatite in vivo. The better effective inhibitors seem to be polyanions, polyphosphonates or predominantly polyphosphates. Salivary proteins and peptides, such as praline-rich proteins (PRPs) and statherin, are powerful inhibitors. This macromolecule seems to stop the precipitation of calcium phosphate (CaP) phases in saliva despite the supersaturation of this specific secretion with regard to HAp. The mechanism of this inhibition was allied to their adsorption on surface of apatite sources[66-69]. However, proteoglycans at low concentrations can prevent or delay apatite generation.
There was a limited knowledge of the crystal structure of biological apatites due to the lack of proper distinct crystals for study[70-72]. However, it had described that the isolated crystals as ofthe natural bones were weekly

crystalline apatite, alike to pulverized intact bone where they had initiated[73]. There are two different kinds of crystallographic structures had been suggested for biological apatites[74-76]: (1) hexagonal and (2) monoclinic with lattice. These both structures could share the same elements calcium and phosphorus with the stoichiometric ratio Ca/P of 1.67. The maindissimilarity between them is the positioning of the hydroxyl functions. In the hexagonal hydroxy apatite, two nearby hydroxyl functions point at the opposite direction, whereas in the monoclinic form—hydroxyl functions.

In the view of the chemical composition of bone, the biological apatites differ from thestoichiometric composition of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ and consist important amounts of several necessary ions such as Mg^{2+}, Na^{+}, K^{+}, citrate, HPO_4^{2-}, carbonate, Chloride, Fluoride ions[77-80] etc. Aqueous biological fluids, these substitute ions changes over time by each and others[81,82] Some analysis like FT-IR (Fourier Transform Infrared Spectroscopy) on the bone mineral reveals that the nanocrystalline apatite is covered with a hydroxy layer covering ions, such as Ca^{2+}, HPO_4^{2-} , CO_3^{2-} , in dissimilar sites of the crystal, which can be considered as dibasic calcium phosphate dehydrate phase ($CaHPO_4.2H_2O$) or octacalcium phosphate $Ca_8(HPO_4)_2(PO_4)_4.5H_2O$ phase[83,84].In several literature reports, the Ca/P atom ratio of biological apatite was either close or lower than that of stoichiometric Ca/P = 1.67[77, 85-87]. Nevertheless,Ca/P > 1.67 has been defined too[88-90]. The exclusive chemical composition of biologicalapatite is exposed by: (a) the lack of anticipatory hydroxyl function, and (b) the existence of HPO_4^{2-}[91].For hydroxyl group, it was stated that only a few percentages of anticipated concentration was noticed in bone[92-94]. The existence of HPO_4^{2-} is attributed to either ionic exchange, or to hydrolysisof PO_4^{3-}.

On further, generally, bone is having four main kinds of cells: a) osteoblasts b) osteocytes c) osteoclasts, and d) bone lining cells. Generally, osteoblasts are bone developing cells. They are initiating from the osteogenic cells differentiation in the tissue which then covers the bone marrow. The preparation of bone matrix by osteoblasts takes place in a couple of steps such as a deposition of organic matrix and then its subsequent mineralization.[95-97] When the osteoblast is completed working, it is thus actually confined inside of the bone after it hardens. This particular stage is known as an osteocyte. Therefore, osteocytes are well developed bone cells and act as stress sensors in the bone matrix. These lining cells help the bone without interfering with other cells functions. Osteoclasts are big multinucleate cells which areaccountable for the collapse of bones. Bone is a complex material wherein CaP is accountable for the hardness, rigidity and high resistance to compression[98,99-100, 101-105].

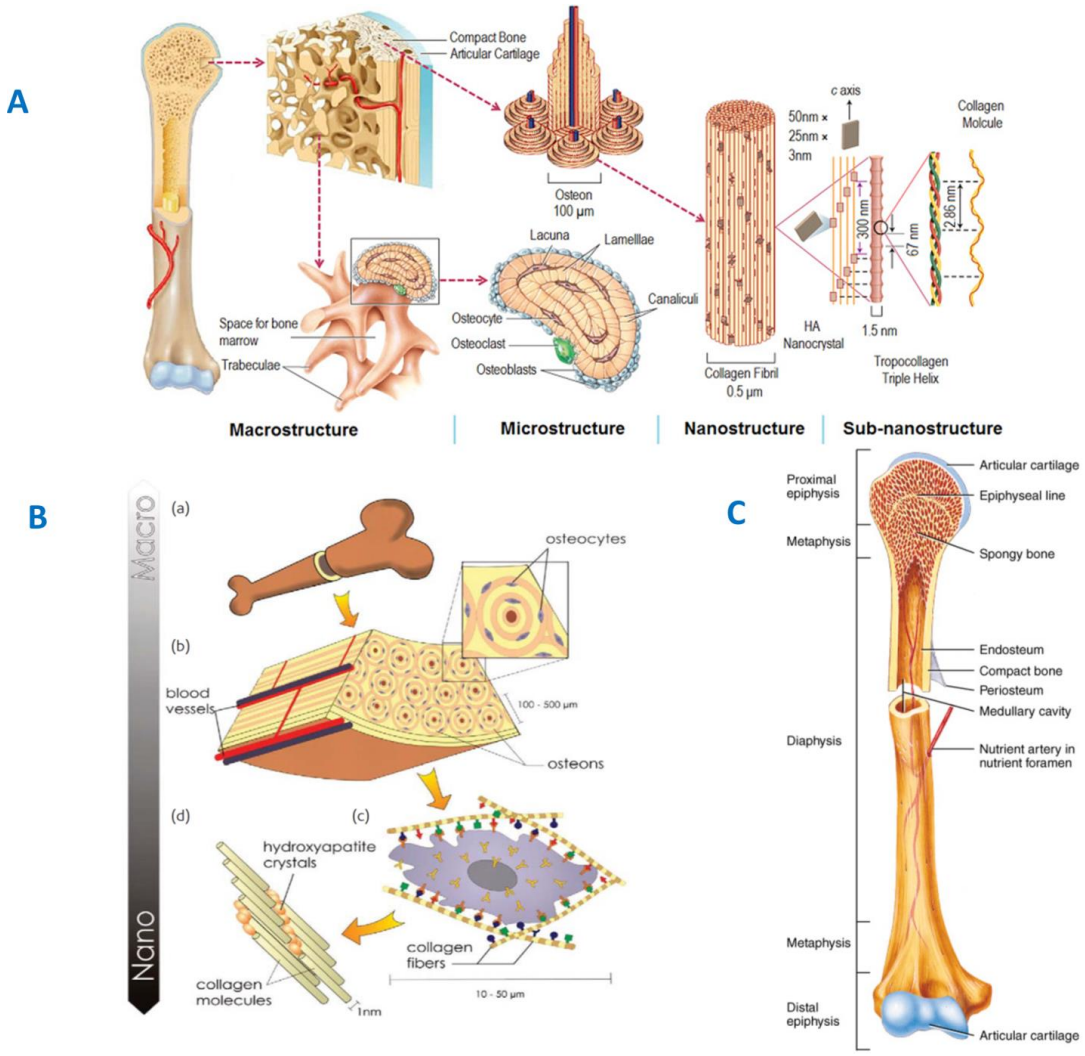


Figure 1. A) Hierarchical structure of bone and bamboo a). In bone, macroscale arrangements involve both compact/cortical bone at the surface and spongy/trabecular bone (foam-like material with ~100-µm-thick struts) in the interior. Compact bone is composed of osteons and Haversian canals, which surround blood vessels. Osteons have a lamellar structure, with individual lamella consisting of fibres arranged in geometrical patterns. The fibres comprise several mineralized collagen fibrils, composed of collagen protein molecules (tropocollagen) formed from three chains of amino acids and nanocrystals of hydroxyapatite (HA), and linked by an organic phase to form fibril arrays. Reproduced with the permission of nature publishing group@2014.Ref.104,B) Hierarchical organization of bone over different length scales. Bone has a strong calcified outer compact layer (a), which comprises many cylindrical Haversian systems, or osteons (b). The resident cells are coated in a forest of cell membrane receptors that respond to specific binding sites (c) and the well-defined nanoarchitecture of the surrounding extracellular matrix (d) (Reproduced with permission from56. © 2005American Society for the Advancement of Science.Ref.102. C)Aclear typical structure of long bone.Ref.9.

3. GF-CaP: Bioorganisms Interactions

3.1. GF and GF-CaP Coatings: Chemistry

In general, graphene oxide was prepared from graphite via two primary methods: (a) top-down method or (b) bottom-up manner. The most common top-down method is Hummer's procedure which could generate a facile protocol for the production of oxidized graphene nano sheets with a better solution process-capacity. Many modified Hummer's methods were developed for the past several decades to produce various nano size shapes or layers with an oxygenated contents[106-111]Fig.2.The common procedure of Hummers' procedure can be accomplished as showed in and in which the graphite in a flake, powder or block form will be chemically transformed or oxidized into a nano layered arrangement, with an aid of sonication, solo or multi-layer oxidized graphene (Called as GO) could be obtained[108-112].This technique was also further customized by elec-

trochemical exfoliation of graphite by using an external electric field; it was regarded as a production of high-quality graphene oxide with fewer defects only in most of the Hummer's techniques [Fig.2b].Graphene was also synthesized from chemical vapor deposition (CVD) method [Fig.2c] and regarded as another representative top-down method, in this procedure, large graphene sheet with diverse sizes and layers can be achieved by applying copper alloy and silicon carbide as substrates[113,114]. Due to high conductivity and the fine structure, the CVD prepared graphene has been measured as the prime method for the production of graphene-based electronic devices. On the other hand, in the bottom-up production of graphene, one of the most thrilling methods is described by the research groups like Fasel and Mullen. These groups reported that atomically accurate graphene nanomaterials (GNMs) with various widths, edge periphery and topologies can be manufactured via surface-assisted polymerization and cyclodehydrogenation of specific precursors.[115]

From the observation of graphene material's chemical and physical properties, it can be divided into three types: (a) pristine graphene (b) graphene oxide (c) reduced graphene oxide. Pristine graphene is a thin layer of pure carbon i.e graphene produced by CVD via the exfoliation of graphite.[116-117] Since these CVD derived graphene materials are having fine aromatic structural properties with a lower number of defects, these graphene nanosheets are problematic to suspend in solutions and consequently not advantageous to use them as nanocarriers or nanomedicine. Conversely, their highly reactive edge surfaces make them feasibly fit for bioelectrodes, for an occasion, the detection of chemical molecules and to bioorganisms. GO is a hydrophilic oxidized structure of graphene or graphene layer has oxygen groups i.e carboxyl, epoxy and abundant hydroxyl groups, especially on its edges and defects as well as hydrophobic sp^2- and sp^3- bonded carbon atoms, hence bring into being a sheet-like amphiphilic colloid.[120]

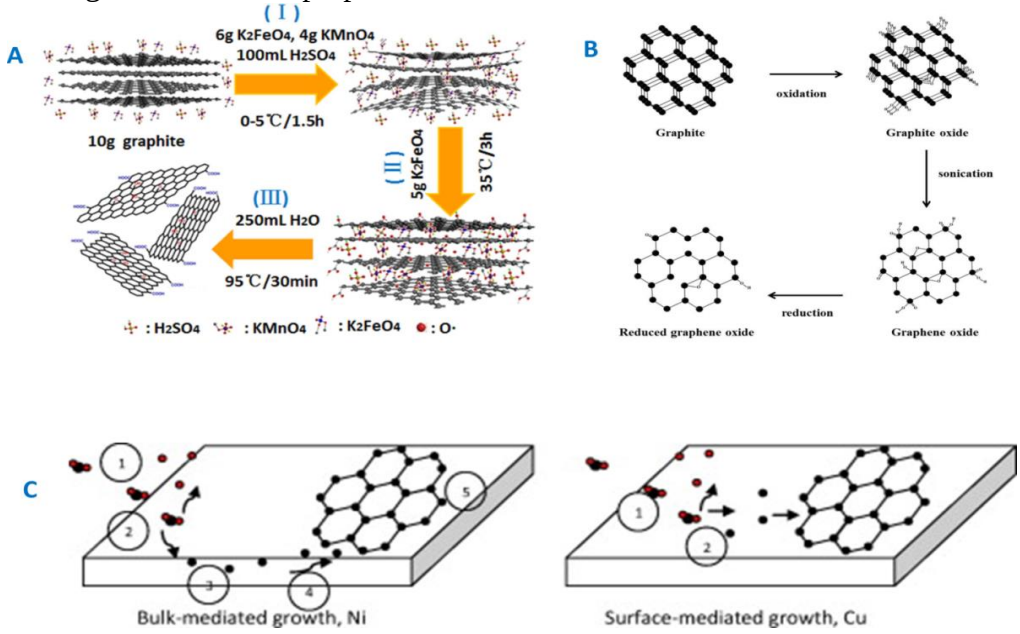


Figure 2. A) Illustration of the preparation of GO based on an newly improved Hummers method.Ref.103 B) 11. Schematic images of the process used to produce reduced graphene oxide (rGO).Ref.9.C)Reproduced with permission of Elsevier copyright @2014.Ref 104.

GO can steadily suspend as amphiphilic colloids in an aqueous and many polar solvents through its hydrophilic groups. Because of the rich residual sp^2-bonded carbon environment on the GO basal plane, GO is gifted of π-π interactions with aromatic moieties. The polar functions, epoxide, hydroxyl and carboxyl acid, on basal plane allow GO to undergo weak or strong interactions with other substrate or organisms depends on their physical and chemical properties.[118]Reduced graphene oxide (rGO), (Fig.2A and 2B) can be achieved by reduction of GO with several chemical, thermal and irradiation approaches. When compared to graphene or graphene oxide, rGO has abundant balanced physical and chemical properties, concerning to solvent dispersibility, thermal, surface chemical groups, optical, mechanical, and electrical performances.[119] Based on the above facts of GF, several people prepared inorganic-based nanocomposites to improve the osteoinductive effect on hMSCs. Various techniques[9,12] could be explored to make these interesting materials (GF-CaP), which are summarized in (Table1). In several cases, GF-CaP biomaterials prepared in high temperature or high pressures have mechanical properties and high crystallinity, such as synthesis, spark plasma sintering and hot

isostatic sintering. Nevertheless, thermal spraying methods typically lower the crystallinity of the HAp coating.

The subsequent compound solutions are suggested to be aged for days to confirm the fully transformed apatite into hydroxyapatite with worthy phase purity as well as crystallinity. For the duration of the synthesis, the oxygen-containing functions on the GO surfaces act as receptor sites for Ca^{2+}via electrostatic interactions; where these anchored Ca^{2+}can in situ react with phosphate ions to get apatite nanoparticles. The essential reaction mechanism has been suggested by Li et al.[12]; the spreading and the microstructures of HA on graphene are mostly affected the amounts and categories of the oxygen functions on the GF based templates and the concentration of the reagents ($Ca^{2+}$and HPO_4^{2-}), solution pH ranges and so on.

In an electrochemical deposition, HApwas involved in dissolving calcium/phosphate ions in a buffer solution with organized pH values and temperature underneath changing electrical current.[120]Once the voltage is applied, Ca^{2+}will transfer to the surface of cathode due to electrostatic attraction and react with the OH^- therein generated by the electrolysis of water, subsequently in the in situ nucleation and growing of HAp on the surface of cathode.[121] Zeng et al.[122] fabricate GO/HApsurface coatings on Ti via this technique; GO was distributed and mixed with the electrolyte for deposition which containsCa $(NO_3)_2$, $NaNO_3$, $NH_4H_2PO_4$, and H_2O_2. The occasioning pure HA coating shows an irregular morphology with shell-like flakes, and the GO/HA complex coating displaysunbroken and porous topography. The upsurge of GO fillings in the electrolyte can increase the HAp crystallinity as well as the bonding strength of the coatings. On the other hand, the applications of HAp for hard tissue grafts are inadequate by the low mechanical strength of associated HAp.[123]

Throughout the conventional sintering procedure, HAp will separate into tri and tetra calcium phosphates at 1000 ºC-130 ºC, and generally, the higher temperatures and long sintering time can reason grain coarsening performance, which may depreciate the mechanical properties[123] of HAp. Graphene/HApnano composites are effectively fabricated by spark plasma sintering. The initial powders that are used for spark plasma sintering can be synthesized by mixing HAp powders/nanoparticles and GF sheets together using a mechanical milling[124], sonication[125 ,126] and prepared by a liquid precipitation way.[127] Different micrometer diameters of graphene are homogeneously dispersed and surrounded within the HAp matrix and situated between the HAp crystal particle boundaries without agglomerations. Characteristic SEMimages of the spark plasma sinteringsamples[9,126] were shown Fig.3.

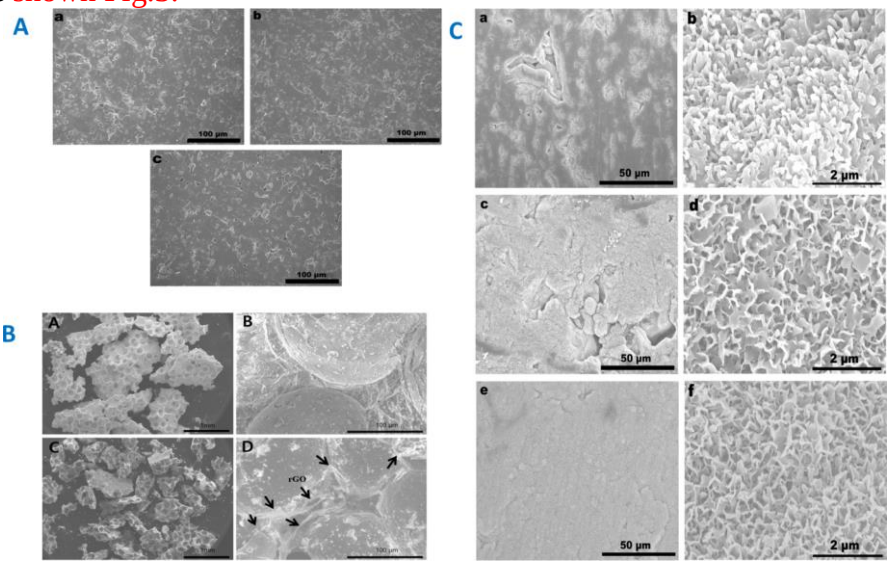


Figure 3.A) SEM images of the surfaces of (a) HA, (b) 0.5 wt.% GNS/HA composite and (c) 1.0 wt.% GNS/HA composite. Reproduced with the permission of Elsevier copyright@2013.Ref.129.B) Field emission scanning electron microscope (FE-SEM) images of biphasic calcium phosphate (BCP) microparticles (magnification: 30x (A); 500x (B)) and reduced grapheneoxide (rGO-coated BCP bone graft materials (magnification;:30 x(C); 500x(D)). Arrow s indicate the rGO nanoplatelets.Ref.9.C) Low- and high-magnification SEM images of apatite formation on HA (a and b), 0.5 wt.% GNS/HA composite (c and d) and 1.0 wt.% GNS/HA composite (e and f) immersed in SBF for 7 days, Reproduced with the permission of Elsevier copyright@2013.Ref.129.

Chemical vapor deposition is a cost-effective and scalable performance to prepare GF films.[128] Novel biocompatible and multicomponent graphene/HA/Au nanomaterials are prepared by using radio-frequency chemical vapor deposition, with methane and acetyleneas the carbon sources[129]. Throughout the deposition procedure, Au nanoclusters are consistentlyspread over HAp particles with diameters of 2 nm to 7 nm and behave as catalyst for graphene manufacture.[129] This research specifies that longer radio-frequency chemical vapor deposition time can conclude in few-layers graphene with greater.[129]

Biomimetic mineralization is an environmental benign technique to prepare bone-like apatite underneath ambient conditions in aqueous locations. Generally, GFs are dipped in an unstable or supersaturated solution with calcium and phosphate ions their concentrations parallel to replicated physiological condition, and apatite was driven as nucleated and precipitate on the surface of those GF-based biomaterials. In the process of mineralization, GO greatly enrich the nucleation and crystallization of HAp, resulting in hybrid uniform GO/HAp coatings with densify fine flake-like HAp nanocrystalline[130]. Typically, GFs are surface modified by bioactive materials to provide the complex with new properties and assist the biomimetic deposition of HAp. The GO can be altered by gelatin to mimic the electrifying proteins in an extracellular matrix (ECM) for a modifiable bone generation, and the existence of gelatin develops the attraction of calcium ions and encourages the nucleation of Hap[131]. As well, GO can also biofunctionalizedby polydopamine[132], carrageenan[133], chitosan, and fibrogens [134,135] to increase the mineralization route. Table 1 show, the most usually used biomaterial composites, nano HAp particles, synthetic polymers and nanoparticles that have been used to get the GF-CaP with worthy biocompatibility and useful biofunctionality. Due to the diverse and exceptional physicochemical and biological properties of GF-CaP, it is supposedto suggest that they can displayplentifuldetailed interactions with tissues, for an example the GF-CaPcombined interface can deliver a more promising microenvironment for cell proliferation and attachment.

Table 1. Various approaches of the GF-CaP related coatings [2,9,12,135-161].

Technology	Categorized procedures	advantages	ref
Synthesis	To synthesize rGO-coated BCP graft material, as-prepared rGO in water was sonicated for 2h, and then mixed with BCP suspended in deionized water at rGO toBCP with various weight ratios. Graphene nanoplatelets were used as the toughening agent. For BCP, first, CDA nanopowderswere prepared bychemical precipitationof $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$ with an appropriate Ca/Pratio.Then,BCPnano powders,amixtureof70wt% HA and30wt% β-TCP were collectedaftercalcininghe obtained CDAat550 ^{0}C. GNPs weredispersed incetyltrimethylammoniumbromide (CTAB) solutionbysonicationfor 1h.Thenthesuspension and BCPnanopowdersweremixedbyb	It is a nontoxic and stable bone graft material. It can increase the bone regeneration better than BCP alone. GNPs/BCP composite are promising bone substitute materials as well as effective additive for toughening ceramics for bone substituents.	[9, 12]

	allmillingfor8h. After beingdried,suchmixturesweretreatedat500^{0}C for1h in argontoremovethesurfactant. Eventually,thecomposites were fabricated byhotpressingthescreenedpowders at 1150 ^{0}C inamultipurposehightemperature furnace under a pressureof30MPain an argonatmosphere for1h.ThecontentsofGNPsinthe compositeswere0,0.5,1.0,1.5,2.0 and2.5wt%.		
Electrochemical Deposition	a) The dispersion of graphene is dripped on copper electrode and carbon film on copper grid by polyethylene terephthalate (PET) dropper, and then dried by air blower. & b) A two-electrode setup operating at room temperature and maintaining a constant cathodic potential of -4.0 V was employed for the electrode placement.b) At room temperature, a two-electrode system with a steady cathodic potential of -4.0 V was used for the electrodeposition process.	The electro-deposition procedure is a facile, environmental friendly and controllable route towards the synthesis of promising graphene-CaP composite for biomedical applications.	[2,120,121, 122]
Spark plasma sintering processing	With a maximum temperature of 1150 ^{0}C and a holding pressure of 40 MPa, graphite papers were positioned between pure HA, 0.5 weight percent graphene nano sheets (GNS)/HA, and 1.0 weight percent and die/punches for simple specimen elimination. The temperature was raised to 1150 ^{0}C in the following minute after a heating rate of 150 ^{0}C/min was applied; this maximum temperature was maintained for three minutes. After that, the samples were cooled in a furnace to room temperature.	It is expected that (GNS)/HA could provide more desirable locations for osteoblast adhesion, as well as creates more nucleation sites enabling apatite mineralization.	[123-126]

Electro spinning	The graphene/HA mixture in organic solution and then subjected to high voltage and being subjected out from the spinnerret	High porosity and connectivity	[127-144]
Self-assembling	Dispersing the graphene oxide/HA into aqueous solution	Controllable porosity and connectivity and good mechanical strength	[145,146]
Thermal Spray	Graphene into the aqueous solution, HA preparation using wet chemical synthesis, spray coated onto the substrate material	Controllable coating thickness and large area deposition and strong adhesion feature	[147]
3D printing	Dispersion of graphene/HA into the specific organic liquid or using 3D printer to synthesize 3d scaffold.	Controllable porosity and connectivity	[148]
Chemical vapor deposition	Au nano particles or clusters are dispersed over HA particles and acetylene and methane are the carbon source using radio frequency chemical vapor deposition	High graphene purity and large graphene sheets	[128,129]
Hot isostatic pressing	Mixing graphene and HA using mechanical milling / ultrasonic dispersion and sintering at high temperatures under high pressures	Ultrafine microstructures , high HA crystallinity and holding fine grain shapes and sizes	[152-155]
Biomimitic mineralization	Graphene family powdered substances were decorated by bioactive materials	Increased osteogenic activities with bone like apatite production	[130-161]

Draw Backs

Inspite of their outstanding biological activities, bioactive GO-CaP materials are still slight delicate and should be even more improved with high toughness nature, factors that considerably limited their appliance in load bearing sites. so, the enhanced mechanical reinforcement through GO still developed with bioactive values and the key gap in the field of biomaterial science should be filled. There are couple of key methods to enhance the mechanical performance of fragile materials: enhancing the fracture energy and dropping the size and severity of defects. As in ceramic developing methods, it may not be possible to keep away the presence of defects completely; in addition, pores are essential to guarantee the show bioactive graphene nanomaterials. Efforts should be put more together on

increasing the confrontation to fracture proliferation[2,3,6]

3.2. GF-CaP Biointeractions and Effects

Understanding the effect of GF-CaP composites on GF-CaP–cell interaction is essential for considering GF-CaP as a potential candidate for bone tissue engineering[9]. Due to the large specific surface area, good obtainability of functional chemical groups, and exceptional interface properties, the GF-CaP possess extremely great dimensions for bimolecular interactions. In recent times, biocompatibility of few layers of GF films conveyed to a variety of substrates was examined using osteoblasts[136]. The substrates were oxidized soda lime glass, silicon wafer (SiO_2/Si stack) and stainless steel. Chemical vapor deposition technique was employed to produce GFs on a copper substrate by using hydrogen and methane as precursors[163]. These studies were also focused on cell attachment as well as morphology and shown that graphene does not have any kind of toxic effect on osteoblasts[136]. The cell adhesion increases with graphene coated material rather than the substrate alone[9, 136]. It appears that GF properties play a leading role in cell adhesion. This study also suggests that layers of GF on bone grafts will be useful for osteoblast attachment and proliferation. Bi et al. have described that the grapheneoxide-calcium phosphate (GO-CaP) nanocomposites notably helped the osteogenesis of hMSCs with enhanced deposition of calcium, which assists their hopeful future in bone repair.[137] For the bone defect repair, S. Wang et al reported a α-tricalcium phosphate (α-TCP) based reduced graphene oxide carbon nanotube cement recently and in which rGO could increase the mechanical assets of calcium phosphate cement effectively with the addition of 0–1 wt%.[138] Chengtie Wu et al prepared that GO-blended β-tricalcium phosphate (β-TCP) biomaterials and proved it in the enhanced osteogenic ability of human bone marrow stromal cells (hBMSCs) instead of pure β-TCP samples both in vitro and in vivo[149]. These scaffolds suggestively improved the activity of alkaline phosphatase, growth and osteogenic gene behaviour compared to the bare β-TCP. The rGO hybridized HAp composites also displayed greater osteogenic differentiation for hMSCs[34]. Jong Ho Lee et al demonstrated that reduced GO-hydroxyapatite composites by adding the 1:1 weight ratio of colloidal dispersion nano particles of rGO with suspended hydroxyapatite (water soluble calcium phosphate) microparticles in DI water, which was enhanced the osteogenic differentiation of hMSCs, when incubated in basal media without any osteoinductive agents[10,34]. In addition the above mentioned nano-sized, functionalized graphene (surface-coated) derivatives, GF coated nanomaterials can also exhibit physicochemical properties as graphene or GO in several ways i.e good biocompatibility, versatile biofunctionality , due to the distinctive and extraordinary specific surface area of 2D planar nanosheet structure, great availability of surface organic functional groups, electrical and mechanical properties.[149] Owing to the diverse and excellent physicochemical and biological properties of GF-coated nanomaterials, it is believed that they can exhibit abundant error-free interactions with proteins, human cells, bacterial and tissues, for instance, the GF-CaP interface can offer a more promising and favorable microenvironment for cell attachment and proliferation. Thus, it is of great prominence to understand these irreplaceable interactions with bioorganisms like stem cells and microbials while we study the biological applications of GF-CaP nanomaterial architectures on bone repair research.

Y.C. Shin et al[139]reported that the hydroxyl groups of the HAp microparticles and oxygenated functions (e.g. epoxy, hydroxyl, carboxyl, and carbonyl functions) of the rGO could able to contribute the stronger adhesion or interconnections in Fig.4A,between HAp microparticles and rGO nanosheets.So, it has been demonstrated that the rGO/HAp composites are stable under cell culture conditions and can maintain their assembly in the cell culture media. He also revealed that rGO/HAp materials prompted significant osteogenic differentiation of MC3T3-E1 preosteoblasts with the associated formation of mineralized nodules from von Kossa staining results (Fig.4B).Von Kossa staining was not noticed in the control cultures without any other composites or particles. This observation of Y.C shin, recommend that the late stage marker of osteogenic differentiation was increased by the synergistic effect of rGO/HAp microparticles in absence of osteogenic factors.[34,140]This observation is also consistent with the recent reports i.e gelatin functionalized GO could becapably used for the biomimetic mineralization of HAp, leading to support the osteogenic differentiation of MC3T3-E1 preosteoblasts.[168]An illustration of the GF-CaP fabrication and its osteogenesis process has been displayed in (Fig.4C).[137]

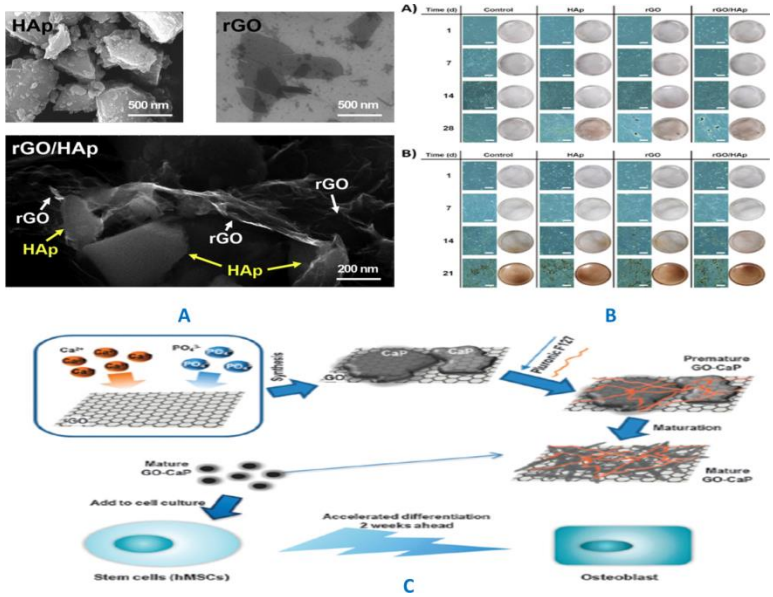


Figure 4.A) Physicochemical characteristics of rGO/HAp composites. (A) FESEM images showed that the HAp microparticles were found to have an irregular granule-like shape with an average particle size of 1080 ± 370 nm and to be partly covered and interconnected by a network of rGO nanosheets which exist as single or few layers. Reproduced with the permission of Elsevier Copyright@2015.Ref.139 **B)** Image of von Kossa stain in MC3T3-E1 preosteoblasts treated with rGO/HAp composites in (A) BM and (B) OM. Dark brown mineralized nodules were observed only at 28 days in BM, while seen even at 21 days in OM regardless of the addition of particles or composites. All photographs (scale bars = 200 μm) shown in this figure are representative of six independent experiments with similar results.Reproduced with the permission of Elsevier Copyright@2015.Ref.139.**C)** Schematic illustration of fabrication procedure for GO–CaP nanocomposites, and subsequent synergistic acceleration of osteogenesis in hMSCs by GO–CaP.Ref.137.

Chengtie Wu et al proved that 3D printed β-TCP scaffolds are extremely porous materials with a homogeneously great pore structure (nearly 500 μm, (Fig.5a)),and the pore walls cover some micropores of 2μm size(Fig.5a) large pore structure (Fig.5b) for the GO to cover whole pore-wall surface of scaffolds (Fig.4e). Which in turn shows that GO coating β-TCP disks have brilliant apatite mineralization ability (Fig.5.f–h), while pure β-TCP does not keep this facility (Fig.5c) and the GO coating β-TCP contain Ca/P ratio of 1.54 (Fig.5g).Thus it is noteworthy to speculate that the enhanced apatite mineralization of this scaffoldoffersnegative chemical groups too from GO, such as COO^-, for nucleation and crystallization of Ca/P ions in simulated body fluids.[137] Ch.Wu anticipated that the above mentioned features and observations are the main reasons for the nanostructured GO coating β-TCP could improve the proliferation and osteogenic differentiation of osteoblasts.[149]GO coating β-TCP bioceramics suggestively encourages the bone defect repairs with the characteristic cell proliferation, osteogenic gene expression of human bone marrow stromal cells (hBMSCs) by motivating in vitro osteostimulation property. (Fig.6d) indicates hBMSCs on GO-coated β-TCP bioceramics grow healthier with higher cell density rather than on just β-TCP disks within short span.This research group also verified the ionic environment of cell culture media with β-TCP and on GO-coated β-TCP bioceramics and concluded that there was no obvious difference about the released Ca concentrations even though P concentrations are somewhat different in β-TCP and GO-coated β-TCP bioceramics, specifying that modification of GO did not muchaffect the ionic dissolution of β-TCP bioceramics. They havebeen recommended that GO coating itself shows significantresponsibility in attracting or increasing the osteogenic differentiation of hBMSCs. Even though there are no research reports on why grapheneoxide has a positive influence on the osteogenic differentiation of stem cells, this research group speculated that the bioactive functions in GO, such as COO^- and OH, might be one of the key factors to straightaway effect cell differentiation through activating the Wnt-related signaling pathway of stem cells. In agreement with this speculation, the previous reportsexhibited that GO can deliver therapeutic via hydrophobic and electrostatic interactions.[9,12,34] In addition, Chengtie Wu et al explained that the GO coating on β-TCP ceramics may also adsorb higher number of proteins from the cell culture media, which additionally increases cell response. Many researchers

over the world also have raised attention in the mechanism of contact of GFs with stem cells, which could add to increase in vitro osteogenesis.

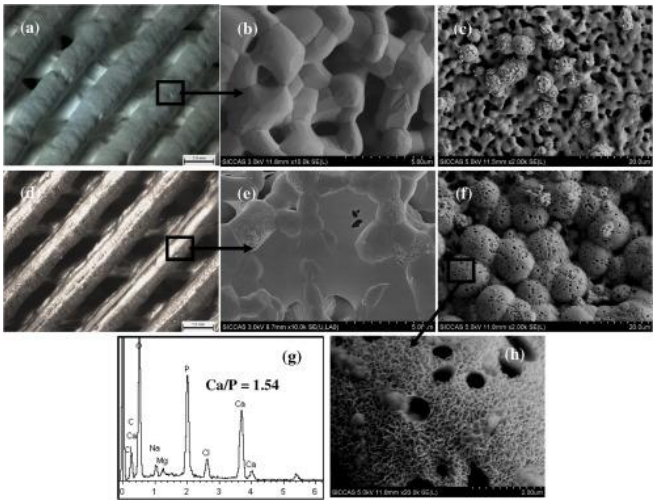


Figure 5.D-printed β-TCP (a) and β-TCP-GRA (d) scaffolds, microstructure of pore walls for β-TCP (b) and β-TCP-GRA (e) scaffolds. After being soaked in simulated body fluids, only a few apatite particles formed on β-TCP scaffolds (c); however, an apatite layer formed on β-TCP-GRA scaffolds (f and h), and EDS suggesting the Ca/P ratio is about 1.54 (g). Reproduced with the permission of Elsevier Copyright@2015.Ref.149.

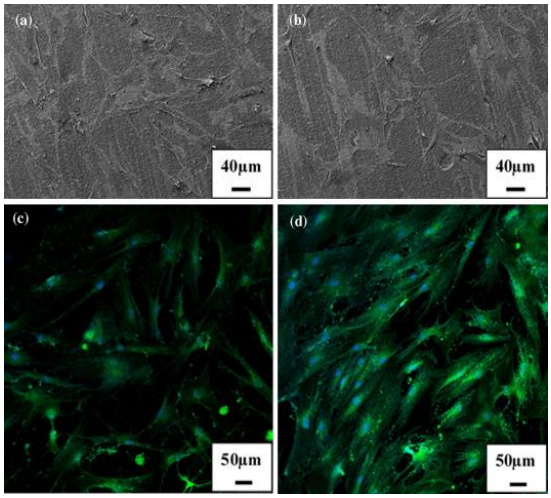


Figure 6. The SEM analysis of cell morphology for hBMSCs on β-TCP (a) and β-TCP-GRA (b) bioceramics after being cultured for 1 day. The confocal fluorescent images for hBMSCs on β-TCP (c) and β-TCP-GRA (d) bioceramics after being cultured for 7 days. Green indicates the structure of cytoskeleton stained by FITC fluorescent dye while blue shows the DAPI-stained cell nuclei. β-TCP-GRA bioceramics could improve cell attachment compared to β-TCP bioceramics. Reproduced with the permission of Elsevier Copyright@2015.Ref.149

Rameshwar Tatavartyet al[137] hypothesized that combining GO with an osteoinductive material can synergistically manage the differentiation of human mesenchymal stem cells (hMSCs) headed for osteogenic lineage. Calcium phosphates (CaP) such as HAp are biomimetic composites that are well-recognized to facilitate the bone formation (osteoconductivity) and to ease osteogenic differentiation of hMSCs (osteoinductivity).However, they prepared the GO-CaP to validate this hypothesis for the osteogenesis and proved that the osteogenic differentiation of the hMSCs via immunofluorescence staining of osteoblast markers ALP and osteocalcin as in Fig.7) &Fig.8. It has been mostly hypothesized that the surface properties of GF nanomaterials such as surface stiffness, nanotopography and large absorption abilityaffect the molecular paths that regulate the destiny of stem cells.[41] Graphene and grapheneoxide were acting as preconcentrators for chemicals, proteins as well as growth factors on their surface to raise cell differentiation.[144] In GO/CaP, GO surface was generallyshielded by CaP nanoparticles, thereforeunapproachable for direct absorption of molecules. The enriched differentiation may in part have got up from the enhanced interaction between the CaP structure on

GO–CaP surface and the intracellular focal adhesion centers of the cells.[135]Moreover, with the incorporation of GO and CaP, GO–CaP biomaterialdemonstratedthe greater stiffness to GO or CaP alone.[145]Such rise in material stiffness could prompt an increasedmechanotransduction effect which has been acknowledged to-control stem cell differentiation and thus might pay to the synergistic improvement in osteogenesis[146].

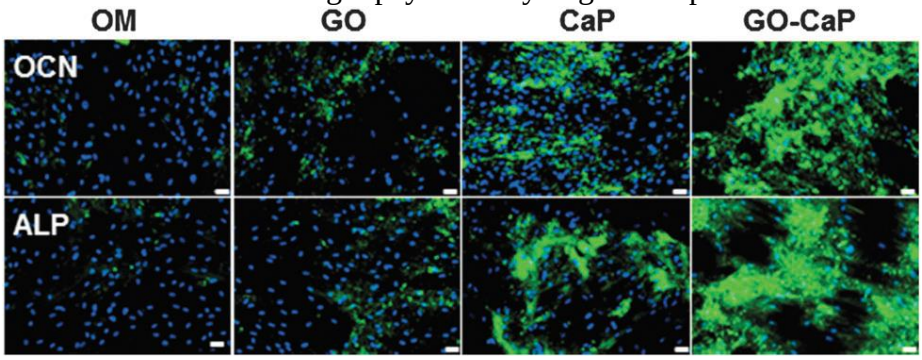


Figure 7. Immunofluorescence staining of hMSC cell culture with FITC labeled (green) osteocalcin (OCN) antibody and DAPI (blue), and Alexa 488 (green) labeled alkaline phosphatase (ALP) antibody and DAPI (blue) after incubation with control osteogenic medium, GO, CaP, and GO–CaP for two weeks. Scale bars represent 20 mm. Ref.137.

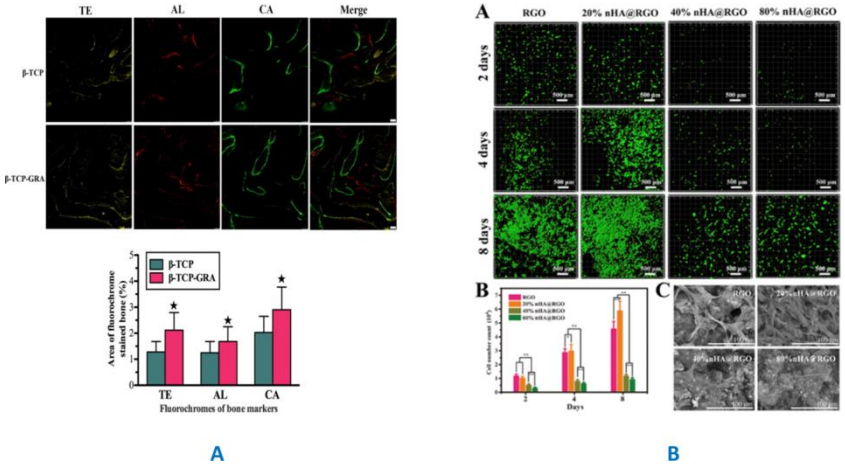


Figure 8. A) Sequential fluorescent labeling; yellow, red, and green color represent labeling by tetracycline (TE), alizarin red (AL), and calcein (CL) for in vivo bone-forming ability of β-TCP and β-TCP-GRA scaffolds after being implanted in the cranial bone defects of rabbits for 4 and 8 weeks (c), respectively. The scale bar is 50 μm. ★Significant difference between the β-TCP and β-TCP-GRA groups ($p < 0.05$). Reproduced with the permission of Elsevier Copyright@2015.Ref.149., B) The rBMSCs proliferated on the scaffolds detected by live cell staining (A) and cell count (B) for 2, 4, 8 days. The SEM image (C) exhibit the rBMSCs growth on the scaffolds for 8 days after seeding. The 3D fluorescence photos were captured using z stack image collection with 40× magnification. Reproduced with the permission of Elsevier copyright@2017.Ref.145.

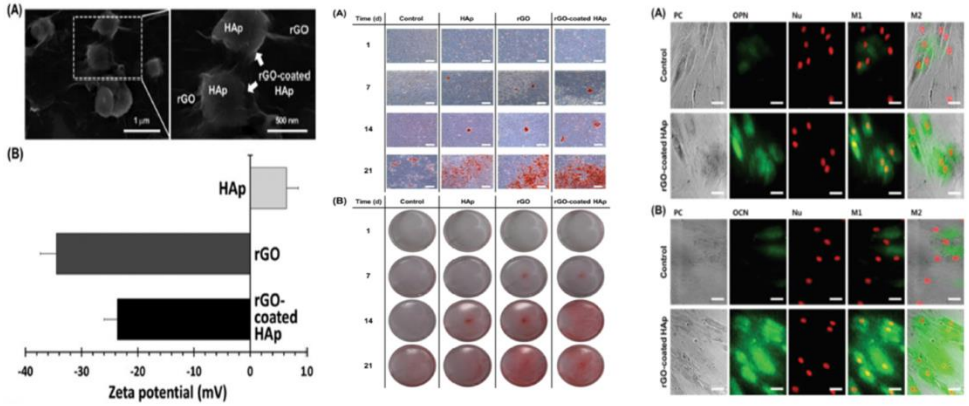
As reported earlier[150], GFs and their derivates consent the attachment of stem cells and stimulate their development and their differentiation to the osteogenic lineage.GO surfaces could be possibly used as delivery transporters for proteins. This probability is supported by the specifics of GO sheets which holds hydrophobic л domains in the core area and ionized functions around the edges of GO. These characteristics significantly increase its interactions with proteins with hydrophobic and electrostatic interactions even in GF-CaP.[9]Previous studies have stated that osteoblasts adhered well and proliferated on the surface of rGO- or GF-HAp hybrid nanomaterials, which proposes that these composites induce 3D adhesion of osteoblast cells and continue cell viability by giving microenvironment alike to that found in vivo.[148-150].ζ potential of rGO-coated BCP composite having stable surface and surface charge of -14.43 mV, which specified rGO-coated BCP bone graft material was designed by electrostatic interactions between BCP and rGO.[9] and this feature of GO-BCP helps on cell growth. The cell growth is also mainly dependent on its structure, size, and concentration.Jeong-Woo Kim et al demonstrated that the cell viability was decreased at rGO concentra-

tions >100 μg/mL, but was sustained above 80% at concentrations <62.5 μg/mL. Thus he suggested from his results, rGO has no harmful properties and it is a non-cytotoxic at concentrations <62.5 μg/mL.[9]

On the other hand, J.H.Lee demonstrated that the osteogenic differentiation of hMSCs was improved by rGO-coated HAp nanomaterials when incubated in basal media in absence of osteoinductive agents. Moreover, he also stated that the osteogenic action mediated by rGO-coated HAp nanocomposites was further increasedwhile cells were cultured in osteogenic medium. An initial coverage of cells to a colloidal dispersion of rGO-HAp material and consequently increased contact with these composites, which in turn enabled intracellular signaling, may proposed as a feasible explanation. However, his results are not clearly proved the mechanism, involved in intracellular signaling pathways. However, his studies supported the rGO-coated HAp composites could be potent factors in helping the spontaneous osteogenic differentiation of osteoprogenitor cells. Thus these rGO-HAp materials might be potential candidates for scaffolds in bone tissue engineering, stimulators for stem cell differentiation and constituents of implantable expedients, due to their biocompatible and bioactive assets.[34]

4. GF-CaP Based 2D & 3D Layered Nano Composites and Influences

Earlier studies specified that 2D GFs can be coated on solid substances of substrates through several physical and chemical methods, and the resulted GF nanomaterial coatings may significantly increase the interfacial properties, such as the mechanical, electrochemical, anticorrosion and biocompatibility so, providing them good application potential for the surface adjustments of biomedical implants.[151]Recent developments have exposed that GF-based 2D and 3D substrates promote the adhesion and proliferation different cell lines such as human osteoblasts and stem cells[152].Human osteoblasts can adhere well to the GF coated materials with a confluent monolayer of normal fibroblast-like morphology, while they exhibit separate round-shaped cells on grafts.[153-156] From the previous reports.[34], it is revealed that, once seeding cells (hMSCs), the cell suspension is incubated with a colloidal dispersion of HAp,rGO nano particles or rGO-coated HAp composite materials in basal media till they were grown as monolayer cultures. Jong Ho Lee et al[34] demonstrated as, it was more advantageous forthe cells which were primarily exposed to rGO-coatedHAp material in 3D culture instead of 2Dplated monolayers and thus the effectiveness of cell contact withthese coating composites was improved, which in turn simplifiedintracellular signaling and succeeding osteogenic activityin hMSCs.Moreover, he also determined the better ALP activity of hMSCs incubated with rGO-coated HAp material in 3D culture was more potential to that in the 2D incubation system since the composite in basal media was treated with as-grown monolayers of cells. Furthermore, the intensity of alizarin red staining was highly increased the 3D incubation system as shown in Fig.9.The same trend was also observed in an immunocytochemical analysis for osteogenic markers, after 14 days of incubation in basal media in absence of osteogenic factors, rGO-coated HAp composites substantially upregulated the expression of OPNand stimulated de novo expression of OCN in hMSCs as seen in Fig.9. Conversely, non-treated cells exhibited few, if any, expression of both the osteogenic markers. This implies that rGO-coated HAp materials could promote the impulsive osteogenic differentiation of hMSCs in a 3D environment. This is consistent with the reports i.e graphene-cell biocomposites increases pellet generation and differentiation of human bone marrow-derived hMSCs headed for the chondrogenic lineage in preconcentrating growth factors[155].The coated graphene oxide also enhanced the calcium deposition in hMSCs.It is suggested that the graphene layer tolerates a noncovalent bridging of protein nutrition and osteogenic growth factors on its flat surface through electrostatic interactions, π-π stacking or hydrogen bonding, thus helping the adhesion and differentiation of hMSCs.Besides, it has been found that the oxygen contents on its surface of rGO-HAp affect the osteogenic differentiation due to the altered interaction between rGO-coated HAp materials and growth factors or proteins.[151,155-156]



A B C

Figure 9.A) Physicochemical characteristics of rGO-coated HAp composites. (A) FESEM images of rGO-coated HAp composites showing rGO sheets surrounding HAp microparticles and covering the HAp MP surface. (B) Surface charges of HAp microparticles, rGO NPs and rGO-coated HAp composites indicating the formation of rGO-coated HAp composites via electrostatic interactions between HAp microparticles and rGO NPs. Reproduced with the permission of royal society of chemistry@2015.Ref.34 **B)** ARS stain and its corresponding extract in hMSCs incubated with a colloidal dispersion of HAp microparticles, rGO NPs or rGO-coated HAp composites in BM. (A) Increased calcium deposits by rGO-coated HAp composites were not related to the cell number (scale bars = 200 μm). (B) There was a notable formation of calcium deposits by rGO coated HAp composites from 14 to 21 days indicating that HAp microparticles and rGO NPs synergistically induce calcium deposition in hMSCs. Reproduced with the permission of royal society of chemistry@2015.Ref.34**. C)** Immunostaining for osteogenic markers for hMSCs incubated with a colloidal dispersion of rGO-coated HAp composites in BM for 14 days. Culture of hMSCs with a colloidal dispersion of rGO-coated HAp composites stimulated de novo expression of the osteogenic markers OPN (A) and OCN (B) (scale bars = 20 μm). These data confirm that rGOcoated HAp composites promote the spontaneous osteogenic differentiation of hMSCs. All photographs shown in this figure are representative of six independent experiments with similar results (PC: phase contrast, Nu: nucleus, M1: merge of OPN (or OCN) and Nu, M2: merge of PC and M1). Reproduced with the permission of royal society of chemistry@2015.Ref.34**.**

On the other hand, the pure graphene multilayer dropped by the CVD technique can offer a favorable biocompatible 2D interface for proliferation, adhesion, and differentiation of hMSCs and neural stem cells (NSCs).Diverse from pure graphene, GF-CaP may display specific interactions with stem cells for the cell morphology due to the rich oxygen functioned flat surface (2D) of the GF materials[9,34,152].Loh et al. find that the modified GO materials can be directly cross-linked at the air-water interface via the dip-coating technique, whereby the coated film shapes as highly wrinkled films.In the interim, the highly wrinkled grapheneoxide film can prompt osteogenic differentiation without any chemical inducers; it is thought that the greatly wrinkled structure shows a driving force en route for an osteogenic differentiation.[149-156]The nanoscale geographical scaffold surfaces can also present anexceptional extracellular microenvironment that impacts the activities of a cell or stem cell, such as a cell shape, growth, adhesionand differentiation.[150]The facilely established large-scale, crumpled graphene or GO layers-based structures are also found to be gifted for guiding cell aligned growth. Since the hierarchical and high-aspect-ratio shaped constructions are fabricated by using the wrinkling and localized ridge uncertainties of GF coating films on prestrained elastomer substarte contents, mechanically stretching the elastomer substrate can effortlesslymanage the patternstructures, thereby facilitating the optical transmittance, wettability, cell alignment and cell adhesion on the substrates. Thus, utilization of electro spinning process to produce nanofibrousmatrix as micro patterned substrates and could be the other facile and inexpensive method for the construction of GF-coated decorative scaffolds.

Besides, for GO-coated materials, water molecules can freely penetrate into GO interlayers while filtration due to the hydrophilic character of the GO sheets, which can later, applied for the water separation from the organic solution. Further cross-linking of GF layers may greatly increase the mechanical characteristics of GO-coated films, in terms of covalent cross-linking or divalent cations (Ca^{2+}) to bridging with the hydroxyl and carboxylic acid groups.[34, 156]

Synthetic 3D architectures of GF-CaP family can generate microenvironments alike to ECM, which thus increases cell adhesion, growth, and differentiation due to the physiological significance of the ECM structure.The design of bioactive scaffolds is an important goal for tissue regeneration.GFs, such as graphene, GO, rGO, and organically/inorganically modified graphene, have been demonstrated to increase the adhesion and growth of mammalian cells including osteoblasts, fibroblasts, stem cells and microbial cells. So, it is significant to explore how the GF-CaP-based 3D scaffolds affect the adhesion and growth of cells. Recently, 3D scaffolds consisting of GF-CaP have been successfully produced by different procedures.[9, 34, 142,144]

5. Emerging Biomedical Applications of GF-CaP Based Architectures

Previous reports revealed that biphasic calcium phosphate was coated at varied levels with GF BCP and connected by GF network.[9,12,34,149] Also, the previous studies have stated that osteoblasts adhered well too and proliferated on graphene-hydroxyapatite hybrid nanomaterials, which advises that these nanomaterials induce the 3D (three dimensional) adhesion of osteoblast cells and sustain cell viability by availing a microenvironment alike to that compared to in vivo.[34,149]Reports revealed that the ID/IG ratio of reduced graphene oxide (1) is little lesser than GF-CaP (1.05) which reveals the increased surface area of the desired nanomaterial. Some studies proved, the cytotoxicity of reduced graphene oxide involves the generation of oxidative stress response[9]too. The previous results[9] suggest GFs have no harmful effects and is non-cytotoxic at concentrations < 62.5 µg/mL. Recent reports also revealing that, after 8 weeks of surgery, entire volumes of new bone (mm 3) were determined by micro-CT, and exhibited that the GF-coated BCP groups showed meaningfully more new bone formation than the selected control group. The GF group and BCP mixture showed the significant total volume of new bone. Some Micro-CT and histometric analysis displayed that the GF group exhibited the highest new bone area. Results also suggesting as the percentage of GF is improved, bone regeneration capability is also enhanced, but when the GF percentage exceeds a definite threshold level, GF cytotoxicity reduces osteoblast viability. The physicochemical characteristics of graphene are likely responsible for its improvement of osteogenic differentiation. The wrinkles and ripples existing on GFs surfaces may affect osteogenic differentiation,[9,34,40,149-162] as these structures support cell wharf and an increased cytoskeletal tension.[125-148] Furthermore, GFs can adsorb biomolecules (dexamethasone) and proteins (β-glycerophosphate) and these could help cell differentiation.[12,34]. Thus, GF-coated BCP bone graft material accelerated fresh bone formation. In addition, adhesion and bioactivity of multilayer GFs have been exploited to improve the detection resolution of several wet whole cells via TEM in an intact culture environment.[157]J.W.Kim et al.[9] have also fabricated graphene oxide-coated biphasic calcium phosphate cell aligners with matching mechanical properties for the bone defect repairs. The designed material is multifunctional and soft cell-culture platform which is able to bring into line the incubated cells and in situ monitoring the physiological behaviors of stem cells during their growth and differentiation. Beyond the in vitro cell imaging, GF-CaP electrodes show abundant potential for in situ cell and tissue engineering. Integrated advantages of biology, electrical, and mechanical stuff are always desirable for materials used in cell and tissue monitoring.[158]

Metal and silicon have been extensively used in fabricating reasonable and conventional prosthetic maneuvers. Nevertheless, their poor chemical-resistance in physiological locations, relatively significant electrical noise, rigid mechanical properties, and high inflammation potential result in abundant limitations in biomedical engineering applications.[159-161] Recent developments in designing GF-CaP[149,152] are focusing on making them as flexible, and bioactive and nanostructured materials as to improve the tissue compatibility and integration. Therefore, GF-CaP based nanomaterials present hopeful forthcoming in succeeding conductive tissue-interfaces, particularly the conductive graphene.[162] Because of the outstanding and exceptional properties, like mechanical stability, electrical conductivity, thermal conductivity, bigger specific surface area, very easier chemical modifications and finally good biocompatibility, it has been believed that GF based flexible could be applied for tissue monitoring claims.

Recent studies have showed that, when GFs are applied for surface coating of implants or membranes, no clear blood and cell toxicity has been detected.[163-165]It is assumed that the reduced “face-to-edge” interaction between the cell membrane and GFs-based coatings would be the key object for their increased cell adhesion and proliferation of modified grafts. Beyond all these mentioned emergencies, spray-coating is one of the best ways to attain a homogeneous and dense surface coating, and Li research group have described the preparation

of the HA-graphene based nanocomposite thin film coatings by liquid precipitation method and the assistance of vacuum cold spraying.[149]The acquired HA-graphene complex coatings retention intact nanostructured morphology and increased spreading and proliferation of osteoblast cells. The electrodeposition of GFs onto conductive substrates is another kind of popular surface coating method.Rhee et al. suggested and reported to coat silver/HA/graphene (Fig.10) porous films on titanium surfaces using electrophoretic deposition.[150]It is also demonstrated that the silver/HA/graphene coated substrates display a diminished amount of surface cracks and increased thermal stability and mechanical property on compared to the graphene-free coatings.The bioactivity examinations revealed that the coated, superior resistance to bodily fluid-stimulated corrosion and enhanced antibacterial activity against E. coil and S. aureus are attributes of the substrate; nevertheless, peripheral blood mononuclear cells have not demonstrated any discernible cytotoxicity.

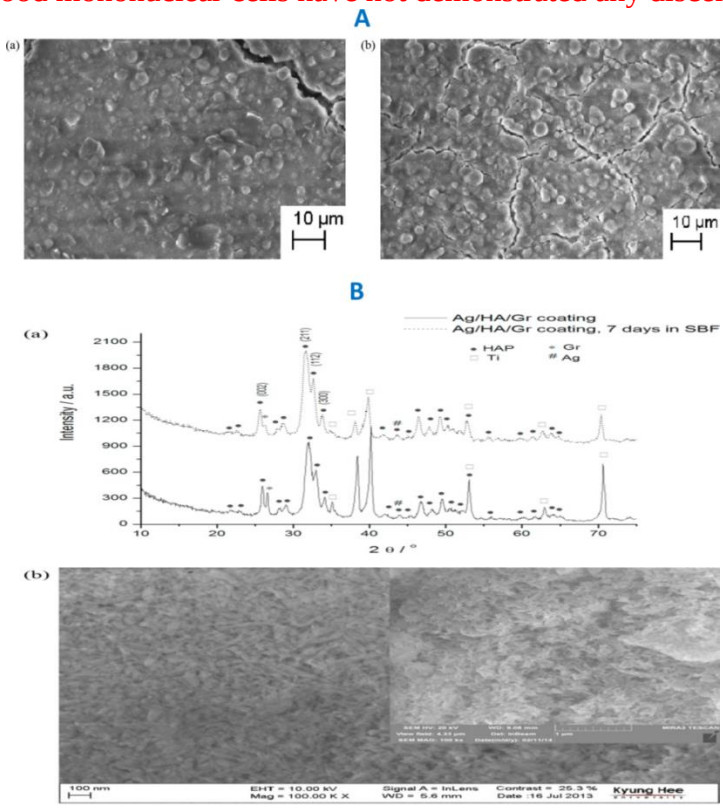


Figure 10.A) FE-SEM micrographs of the Ag/HAP/Gr (a) and Ag/HAP (b) coatings, magnification 1000× **B)** XRD patterns (a) and FE-SEM microphotographs (b) of the Ag/HAP/Gr coating before and after immersion in SBF (inset: Ag/HAP/Gr coating, 7 days in SBF, 37 °C). Reproduced with the permission of Elsevier Copyright@2015.Ref.108.

In the same way, highly biocompatible composite coatings of HA-GO [125], HA-gelatin-GO[166]and HA-chitosan-GO[9,10,124, 142 ,167]designated assemblies on titanium have also been accomplished. These materials display good biocompatibility for the period of incubation with MG63 cells (Fig.11c).Once an incorporation of GO, the coated films turned out to bea porous, additional corrosion resistant, and extremely cell compatible.

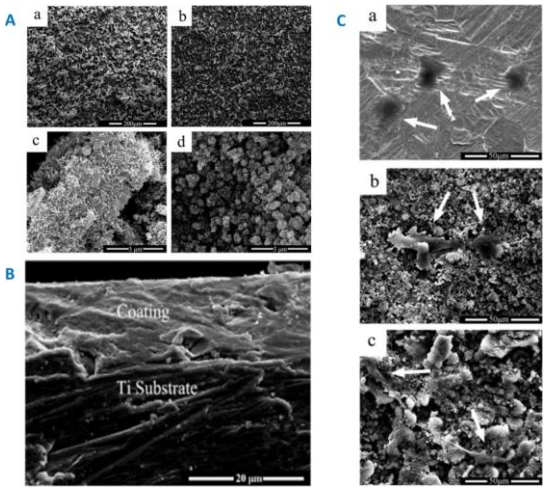


Figure 11.A) Surface morphologies of the pure HA coating (a) and 100 µg/mL GO/HA composite coatings (b) in low magnification SEM images; Surface morphologies of the pure HA coating (c) and 100 µg/mL GO/HA composite coatings (d) in high magnification SEM images**. B)** Cross-section of the 100 µg/mL GO/HA composite coating.C) SEM micrographs of MG63 cells cultured on pure Ti (a), pure HA coating (b) and 200 µg/mL GO/HA (c) composite coatings for 24 h. The white arrow heads point to the cells. Reproduced with the permission of Elsevier Copyright@2016.Ref.137

Even some other calcium based HAp has been broadly used as coating resources for metal implants to increase biocompatibility and accelerate early cellular relocation.Conversely, the deprived fracture toughness and wear resistance limited their long-standing usage once the implantation. J.W.Kim et al. alternatively directed that the GF-supported calcium phosphates could be used to reinforce the mechanical properties of any kind of calcium based bioceramic coatings.[9]It was also observed that the GO coating on calcium phosphates can induce rapid production of HAp layer (Fig.12), thus generating better adhesion and growth of stem cells.

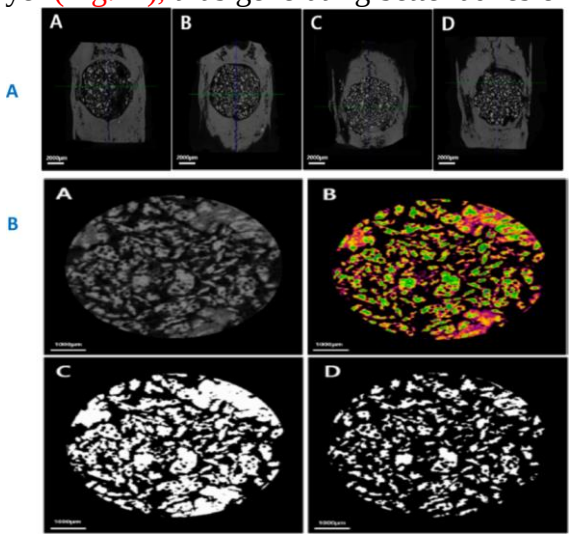


Figure 12.A) Micro computed tomographic images. A) Control group ; B) rGO2 group ; C) rGO4 group; Ref.9. D) rGO10 group **B)** Micro-computed tomographic (CT) images of regions of interest. (A) Reconstructed image; (B) Color image (yellow and green-bone graft material and orange and purple-new bone); (C) Total bone (bone graft material and new bone) image; (D) Bone graft material image.Ref.9.

The GF-CaP biomaterials have been proven to function as effectual coating composite materials compared to pure HA coatings alone. The addition of GF materials into calcium phosphate coatings can minimize the potential of surface cracks with enhanced adhesion strength and resistance to body fluid induced corrosion. Furthermore, the GF-CaP coating reveals much higher viability and cell adhesion compared to the HA uncoated and coated nano metal substrates. Recently, Weibo Xie et al[168]reported, the mechanically robust 3D graphene–hydroxyapatite hybrid bioscaffolds (GHB).As shown in Figure 21 a–c, the surface of the GF scaffold was mineralized with electrodepositing circumstances of -1.4 V and 30 ^{0}C for 30 min. The related SEM images at dissimilar scale bars (200, 50, and 5 µm, respectively) display that the hydroxyapatite or cal-

cium phosphate composite is conformably covered on the surface of micro-branches within the graphene foam substrate. Though the pore size of graphene foam is somewhat reduced, there is no hole-blocking phenomenon that occurred. The high magnification image demonstrates the lamellar-featured structure of the calcium phosphate composite on multiscale with the usual thickness of 3.6 ±0.5 μm. Furthermore, the effect of electrodepositing conditions on the morphologies of calcium phosphate composite was further examined at dissimilar temperature of 60 ^{0}C (-1.4 V/60^0 C for 30 min), as demonstrated in Figure 2d–f. Fascinatingly, besides the same uniform and laminar deposition of CP composite, there are important changes of the morphologies with more regular and smaller grain size displayed, leading to additional enhancements of density and toughness for the mineralized graphene foams. As shown in Figure 21B (a-c), the TEM images displayed that calcium phosphates possess flake micrographs with laminar like crystal structures by various sizes, which is consistent with the results obtained by SEM characterizations as presented in Fig.13A. These materials were then further checked in their bone related applications which can show an improved osteoconductive and biocompatible performance.

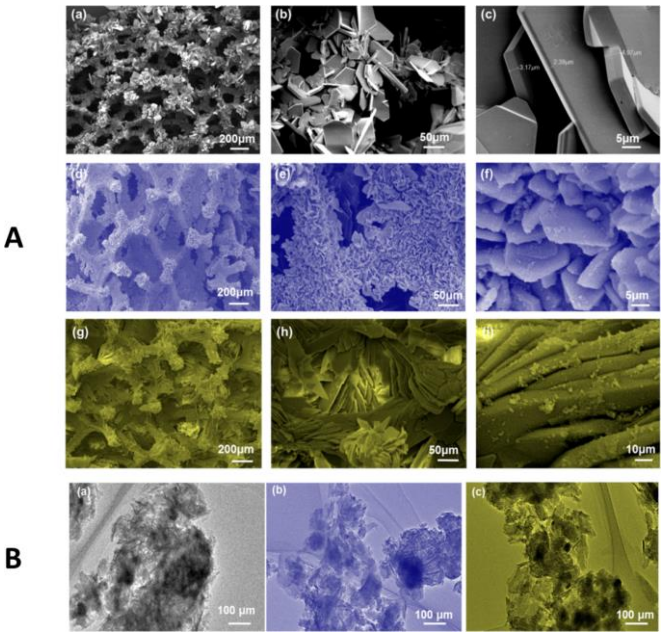


Figure 13A)SEM images of graphene–hydroxyapatite hybrid bioscaffolds (GHBs) for different electrodeposition conditions. (a–c) 30 ^{0}C and -1.4 V for 30 min. (d–f) 60 ^{0}C and -1.4 V for 30 min. (g–i) 30 ^{0}C and -2.1 V for 30 min.B) TEM micrographs of GHBs with similar deposition conditions. (a) 30 °C and −1.4 V for 30 min b) 60 ^{0}C and -1.4 V for 30 min. (c) 30 ^{0}C and -2.1 V for 30 min.Adapted from ref.168**.**

The health status and cytostatic activities of MC3T3-E1 cells were also evaluated by acridine –orange-ethidium bromide (AOEB) double staining assay. Studies have exposed that AO reagents can only enter into the living cells with the fluorescence appearing as green color, whereas EB only enters into dead cells to be orange-red [168]. Figure 23 displays the AO–EB staining fluorescence images of MC3T3-E1 cells co-cultured on 2nd day and 4th day, where cells in the all images are almost green(Fig.14). The total cells within both 3D graphene foams and GHBs rise with the prolongation of culture time (2–4 d). Relatively, the 3D GHBs determine the higher densities of cells on the surface than that of the GF all over the whole incubation period, which is consistent with the MTT results [168]. Such prior actauthenticates that both kinds of scaffolds do not have cytotoxicity and can offer compatible substrate for cell adhesions. So, it is essential to show that 3D GHBs are more capable of endorsing the proliferation of MC3T3-E1 cells.

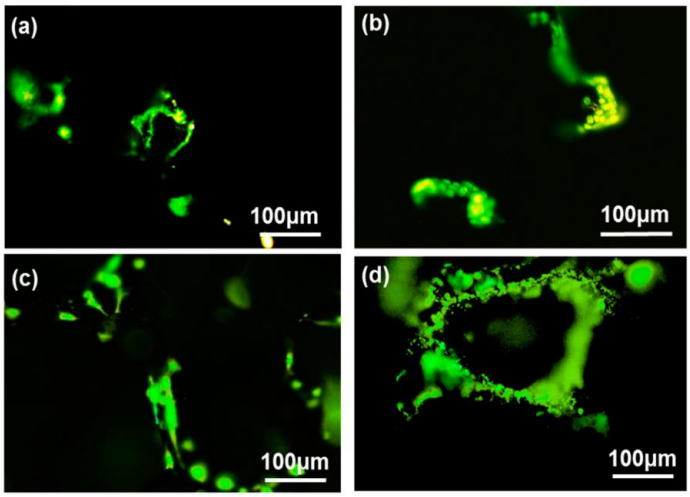


Figure 14. Flourescence microscope images of the MC3T3-E1 cells after acridine orange-ethidium bromide (AO–EB) double staining. (a) and (b) 2nd and 4th days on 3D GF, respectively. (c) and (d) 2nd and 4th days on 3D GHBs, respectively.Adapted from ref.168**.**

Conversely, the GF-CaPs materials possess some disadvantages such as using expensive experimental set ups eg. Autoclaves. Inability to observe the crytal as it grows in synthetic process. In situ setting with particle leaching has numerous drawbacks: a) because the porogens inside the materials have limited acquaintance to body fluids, the solubility of the particles or degradation may be compromised, which might lead to limited porosity. b) The in vivo dissolution of some particles may affect the hyperosmosis. c) Some porogens may raise the paste viscosity and obstruct the injectability of GO-CaP. Therefore, pre-fabricated calcium phosphate scaffolds have been established to allow more gentle control of the setting procedure and macroporous GO-CaP construction of the scaffolds before in vivo implantation.Hence, to resolve the drawbacks, recently, three-dimensional (3D) printing has rapidly advanced to allow the fabrication of bone regeneration scaffolds. 3D printing is a preservative manufacturing procedure in which geometrical data are used to generate 3D structures by placing materials layer by layer. 3Dprinted graphene based CPC scaffolds are preferred over customization to meet the precise needs of each defect.The benefits for clinical applications include tranquil adaptation and fixation, minimized surgical time, satisfactory esthetic results and minimal waste products.So, new tissue engineering methods utilizing CPC scaffolds with co-culture and tri-culture represent exciting alternative strategies that warrant further research for continued improvement to achieve wide clinical applications.

6. Standpoint and Future Directions

GF-CaP have been engaging more and more attention as an evolving stand in the fields of material science, chemistry, biomedical engineering due to their superior mechanical properties, higher surface area, outstanding thermal and electronic properties, as well as easy chemical modifications. Rather than their uses in nanomedicine for phototherapy and drug/gene delivery GF-CaP have shown tremendous interaction and adhesive possessions for protein, microbial and mammalian cells, which make GF-CaP hybrid designs potential stages for multifunctional biological applications.In this review, we have concise the recent developments in the construction of GF-CaP architectures for biological applications in cellular signal detection, stem cell engineering, implant coating, bone tissue regeneration. It is believed that the design of GF-CaPs hybrid architectures have an auspicious future and will draw benefits across a broad range of research fields.

Inspite of the great efforts that have been taken to construct GF-CaP-based nanocomposites, such as 3D printing scaffolds, oriented porous hydrogel, thin film coating, yet challenges exist.Additionally, more research is required to further disclose the inherent physical and chemical properties of GF-CaPs based biomaterials and to propose more tunable approaches in order to achieve nanostructured film coatings and to build hierarchical porous sprays. On the other hand, extra potential applications for this GF-based CaP coated hybrid architectures ought to be revealed. Many applications are still restricted to bone recovery. Recent advancements have shown that electrically conductive and mechanical properties endow GF-CaP-based composites with a promising potential for bone tissue engineering. GF-CaP coatings large surface area confers them the ability as carriers to concentrate growing factors and many other kinds of ECM proteins to uphold

cell adhesion, thus succeeding the cells' existence and proliferation in stem cell therapy in bone research. The GF-CaP hybrid architectures have been attained in dissimilar 2D and 3D forms, like micro/nanofabrication, multilayer coating and free-standing films, and even 3D foams. These materials have revealed great potential for many applications instem cell and tissue engineerings. Both the extraordinary mechanical and electrical properties of GF-CaP coatings allow them to regulate stem cell growth and differentiation into aimed tissues, especially bone. Nevertheless, the underlying signaling pathways and mechanisms for the differentiation and adhesion of stem cell on GF-CaP based substrates are yet not evidently understood. Additionally, studies are also needed to uncover the potential principles at the cellular/sub cellular level and to offer new evidence for stem cell-based therapies. Besides, the design of GF-CaP hybrid substrates, 3D scaffolds or thin films, which have been interfaced with dissimilar nanomorphologies, mechanical and electrical stimulations, are both interesting and compulsory to further disclose the nanostructure−stem cells interactions.

Since GF-CaP is non-biodegradable supplies, long-termand thorough histological studies of a broad range of organs and tissues will be significant to assess health risks,which is unsafe before these GF-CaPs can be used for implantable applications.More importantly, dissimilar aspects of the graphene based nanomaterials such as functionalization methods, and bioactivation of calcium phaosphate coatings and etc. were explained in detail. Also, numerous outstanding properties of the bioactive GF-CaPs and their research choices as well as the development potential and visions were discussed. Furthermore, this survey challenged the possibility of the graphene based scaffold surface that act as interesting signals for bone cells to promote the bone regeneration procedure.Nevertheless, along with detailed in vitro representation of scaffolds, more emphasis should be found on their evaluation in vivo with respect to inflammatory responses, regenerative potential and biocompatibility.

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