https://doi.org/10.48047/AFJBS.6.16.2024.433-446

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Graphene-Calcium Phosphate Nanocomposites for the sustainability of Bone repair and its Biological Properties

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Volume 6, Issue 16, Nov 2024

Received: 23 Oct 2024

Accepted: 20 Nov 2024

Published: 05 Dec 2024

doi:10.48047/AFJBS.6.16.2024.433-446

Abstract

Composites of calcium phosphate (CaP) with the graphene family (GF) show promising potential as materials for bone regeneration. GF nanomaterials can undergo physical and chemical modifications to mimic biological systems, resulting in their exceptional thermal, mechanical, and electronic properties due to their stability. These GF composites, which are biocompatible and coated on calcium phosphate, can improve stem cell growth and differentiation, as well as tolerate proliferation into different lineages or interact with bioorganisms. Enhancing the progress of CF-CaP materials and their interactions with osteoblasts, both physicochemical and biological, is crucial for accelerating the healing and repairing of significant bone imperfections or defects. This article discusses the impact of CF-CaP and outlines recent advancements in the creation of CF-CaP structures for bone regeneration with multiple functions. We have additionally examined the usual biological uses related to these bioactive nanocomposites based on CF-CaP. Moreover, emphasis will also be placed on future perspectives and changing challenges. Because there is a lack of comprehensive reviews in this developing research area, this review would attract significant interest and create new opportunities in a variety of disciplines.

Keywords: Graphene-Calcium composites; mechanical properties; osteogenic differentiation; bone tissue properties

1.0 Introduction

Bone tissue regeneration is a top priority for enhancing the speedy healing and repair of significant bone defects resulting from skeletal issues, tumor removal, fractures, and infections. This article focuses on the use of graphene family (GF)-calcium phosphate (CaP) materials, such as Graphene (G), Graphene oxide (GO), and reduced graphene oxide (rGO), as bone substitute materials for various bone procedures like repair, replacement, regeneration, or augmentation[1-5]. GF-CaP biomaterials have received significant attention because of their bioactivity. Therefore, this evaluation will also include the impact of the surface composition of calcium phosphate along with its physical and chemical characteristics following the introduction of GF nanomaterials[6,7]. The growth in this area requires the use of substrates that support cell adhesion and differentiation.[8] Numerous diversity materials have the ability to trigger, begin, and withstand a sequence of intricate actions that can lead to cell differentiation and osteogenesis. Generally, collagen may have suitable surface chemistry for cell differentiation and growth, although it lacks good mechanical properties and is prone to immune response. Hydrogels, which have adjustable physicochemical properties, could potentially guide stem cell fate in a positive manner. However, the limitations of cell-specific bioactivities at their boundaries can make it challenging to create large structures because a highly interconnected network is necessary, which can impact cell behavior. Therefore, materials with important features are essential for supporting cell growth and promoting differentiation, showing great promise for stem cell research.

Graphene, along with its derivatives GO and rGO, are gaining more recognition in the field of biomedical applications due to their exceptional characteristics like high surface area, strong mechanical and electrical properties, and ease of chemical modification. Graphene is particularly structured with sp2-bonded carbon atoms. Furthermore, Graphene is the thinnest and most robust single layer and lightest substance that can exist independently. The first method used to isolate individual graphene nano sheets from graphite involved using Scotch tape to peel them off. Other methods for preparing graphene include growing it from a raw carbon source, cutting open carbon nanotubes, using sonication, and reducing carbon dioxide or graphite oxide. Furthermore, GO exhibits exceptional hydrophilicity due to the incorporation of oxygen-based groups like hydroxyl, carbonyl, and carboxyl on its surface. Due to its excellent water dispersibility, high aspect ratio, and desirable mechanical properties, GO has emerged as a top contender for enhancing cement-based materials.

2.0 GF-CaP: preparations and Bioorganisms Interactions

In synthesis, rGO-coated BCP graft material was prepared using the BCP microparticles i.e mixture of HAp and β-TCP (3:7 by weight). The readily prepared

rGO in DI water was subjected sonication for 2h, and then mixed together with BCP, suspended in DI water at rGO to the BCP weight ratios of 2:1000, 4:1000 and 10:1000. The rGO-coated BCP material was gained by robustly mixing colloidal diffusions of rGO and BCP microparticles for a while (10 min) and was slow air-dried at RT for overnight[9-10]. Nano HAp particles are effectively fabricated on GO[6,7], chitosan modified[7] GO and rGO surfaces[9] using in situ synthesis approaches. Generally, as shown in Fig.1, GF based powders[7] are initially dissolved and exfoliated in deionized (DI) water by sonication to attain a uniform mixture; later $Ca(NO_3)_2$ is added into the GF based solutions by stirring for a preferred time; subsequently, the pH of the suspension is changed to 9-10 using ammonia solution, and $(NH_4)_2HPO_4$ was added into the mixture[7]].The SEM and TEM images of the physicochemical characteristics[11] of rGO/HAp shown in the Fig.2.

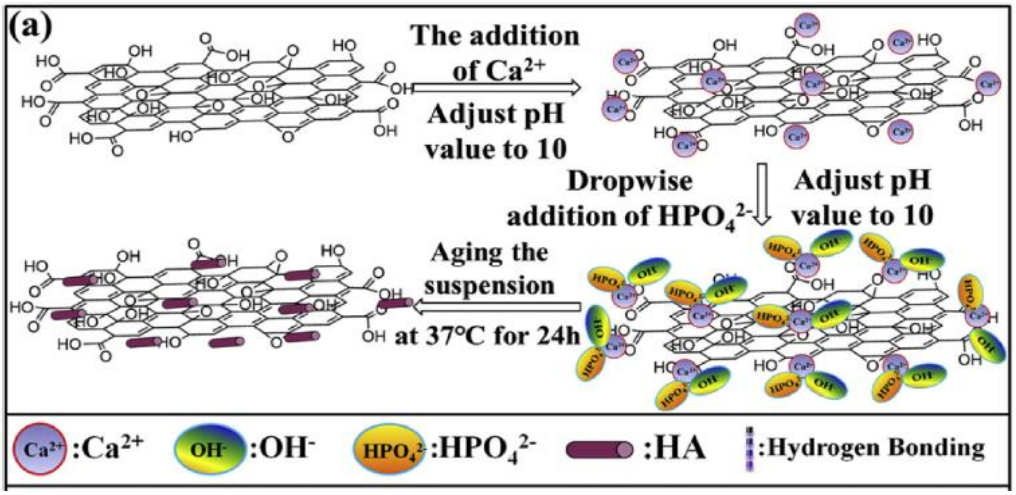

Figure. 1. The proposed in situ synthesis mechanism of HA on pristine GO sheets. Ref.7. Reproduced with the permission of Royal Society of chemistry@copyright 2012.

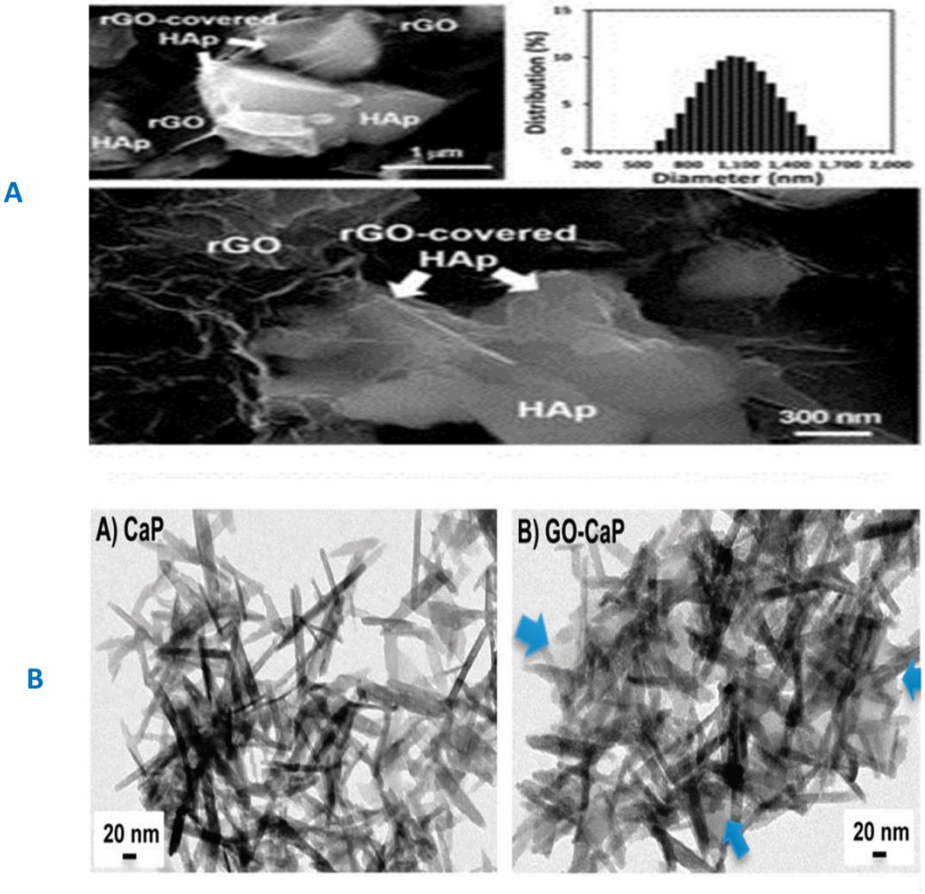

Figure 2. A) FESEM images of the rGO/HAp NCs show that the morphology of the HAp MPs was irregular-shaped granules with a mean particle size of 960} 300 nm as well as that HAp MPs were partly covered and interconnected by an network of rGO NSs. Ref.No 10, B) TEM images of mature (A) CaP nanoparticles and (B) GO–CaP nanocomposites at 2 weeks after fabrication. Blue arrows on (B) point to the edge of underlying GO sheet. Scale bars indicate 20 nm. Ref.11.

In the electrospinning technique, electrospinning pays an electrical field formed under high voltage to force out the polymeric liquid from the spinneret, causing in a polymeric fibrous and porous scaffolds on the collectors[12]. Previous reports[13] explained the preparation of porous polylactic acid (PLA)/HAp/GO scaffold by using electrospinning technique and SEM images of the composite material were shown Fig.3. In a self-assembling technique, GO offers a facile and effectiveprocess to produce GF-based macrostructures. As shown in Fig.4A, grapheneoxide and HAp nanoparticles (nHAp) mixed together were sonically in ice bath which results in a homogeneous suspension, and later the mixture was heated at 200 ^{o}C for 3 h to prompt self-assembly [14]. This method has reduced the grapheneoxide to rGO without using any reductant substance and organic solvent, which could extremely decrease the cytotoxicity of the composite. The SEM morphology images and the EDS data clearly differentiated the substrate and desired final material (Fig.4).The SEM images (Fig.4A), revealed that the displayed scaffold was porous structure (diameter in range of 20–100 μm). It was also evidently observed that the amplified mass ratio of nHA could alter the morphology of scaffold. Thus, the mass ratio of nHA to GO was a significant parameter affecting scaffold assembly and should be well determined to ensure the good biocompatibility for bone defect repair[14]. The

spot EDS analysis (Fig.4B) on SEM images presented that the amount of carbon element on the surface of scaffold reduced with the rise of nHA ratio, indicating that nHA was integrated on the surface of graphene nano sheets.[14] Thermal sprayed HAp and HAp-based coatings have been fruitfully used on commercially accessible Ti-based orthopedic grafts, having the benefit of high deposition rate, decent bonding strength and adjustable coating thickness[15]. This procedure contains, heating the HA powders to melting stage at high temperature, which may reason for the breakdown of HA and show detrimental properties on the coating biocompatibilities. Consequently, other reports[16] altered vacuum cold spraying as a substitute to prepare GF/ HAp nanostructured coatings at RT[16]. The GF/ HAp powder material is made by wet chemical method, and the sprayed coatings have a measurable thickness and show modest pasty strength and fracture toughness, with graphene uniformly embedded in HAp matrix[16]. Three dimensional (3D) printing is a greater additive manufacturing system to print scaffold with tailored shape, precise chemistry and porosities and displays great potential for its application in bone tissue biomedical engineering[17]. Even though bone has self-healing capabilities, the heavy bone loss or injury cannot be restored totally and naturally. A matrix or scaffold materials should be incorporated to support this healing course. From the previous reports[18] , there was a manufactured GO surface modified β-tricalcium phosphate (β-TCP) framework by, first using 3D printing technique and then soaked the β-TCP framework into GO/water suspension for the developments of in vivo osteogenesis.

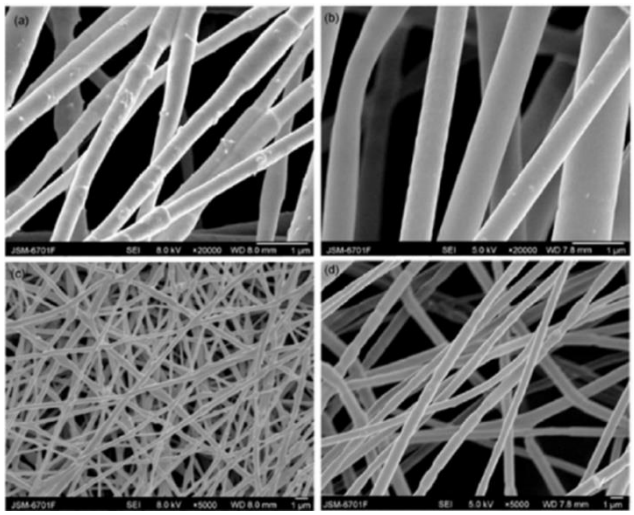

Figure 3. SEM images of PLA/HA/GO nanofibers with high magnification (a) and low magnification (c), electrospun PLA with high magnification (b) and low magnification (d).Ref.[13].

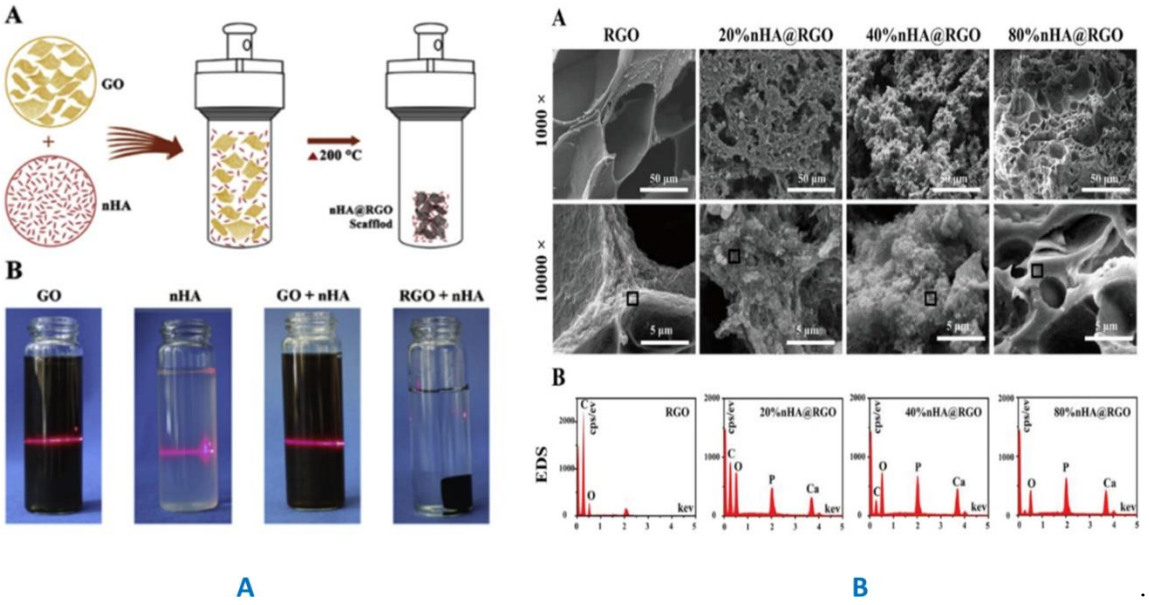

Figure 4. A) (A) Schematic showing that the self-assembly of RGO and nHA to form a porous RGO scaffold for cranial bone defect reconstruction; (B) Tyndall effect of before and after reaction. B. (A) SEM and (B) EDS analysis of the nHA@RGO scaffold with the different nHA loading ratios, the black box on the photo was the sampling area of the EDS.Reproduced with the permission of Elsvier copyright@2017.Ref.14.

The GO coating was synthesized on the surface of 2D disc circles and 3D scaffolds of β-TCP ceramics by soaking ceramic scaffolds in GO-H_2O suspension in combination with following heat treatment. As displayed in Fig.16a, surfaces of β-TCP disks are flat and smooth. Nevertheless, later, being coated with grapheneoxide, the surfaces turned rough with diverse GO nanosheets (Fig. 5b and d). The β-TCPs are white, and afterward modification with grapheneoxide, the whole β-TCP disks are gray (Fig. 5c), representating that GO can covers the total surface of β-TCP disks. The characteristic C1s peaks of XPS agreeing with C-C, C=O and carboxyl (COOH) and are distinct for GO/β-TCP (Fig. 5e and 16.f) which thus subsidize to the better hydrophilicity on the disk surface (Fig. 5h). Once the GO modification, there was no phase has been changed for β-TCP bioceramics (Fig. 5g). As discussed earlier GO-Coated-β-TCP disks enhance the hBMSCs grow better with higher cell concentration than those on β-TCP disks [18].

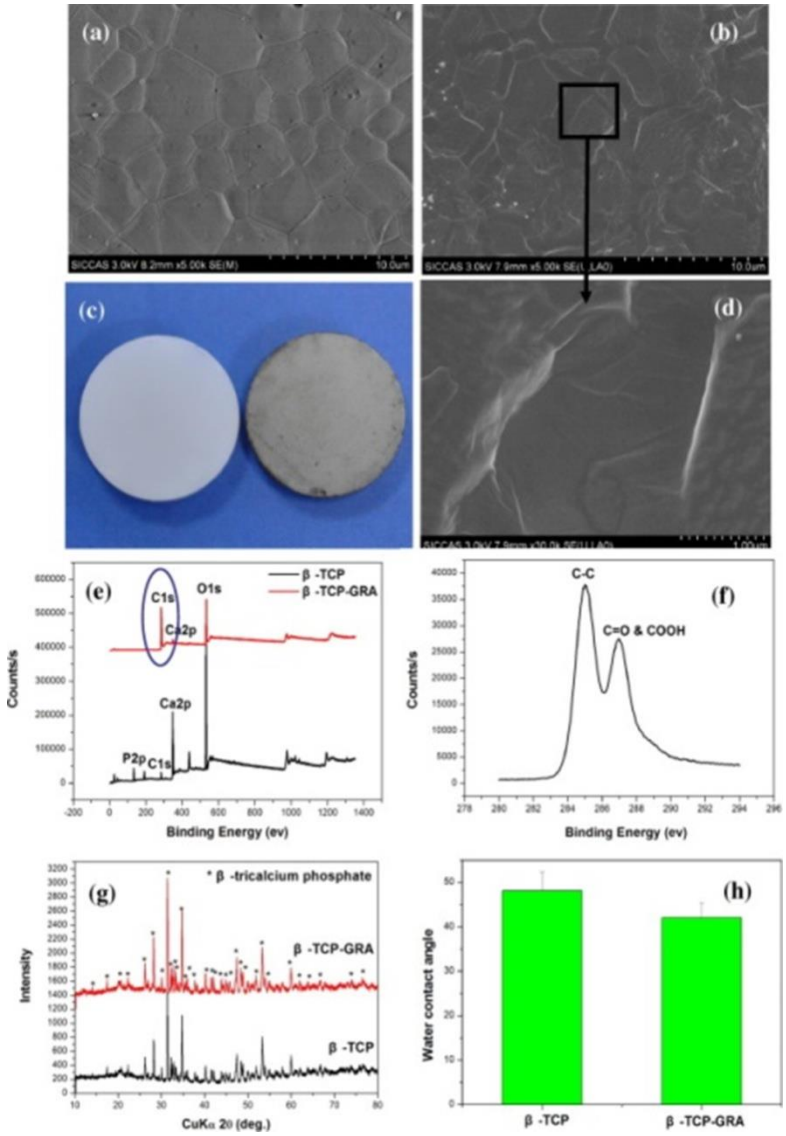

Figure 5. a) SEM for the prepared β-TCP (a) and β-TCP-GRA (b and d) bioceramic disks, (c) the whole morphology of β-TCP (left) and β-TCP-GRA (right) disks, (d) the higher magnification image. Arrows point to graphene oxide. (e) XPS analysis, (f) the C1s peaks for β-TCP-GRA bioceramics, (g) XRD analysis, and (h) the water contact angle (degree) for the prepared β-TCP and β-TCP-GRA bioceramic disks. Reproduced with the permission of Elsevier Copyright@2015.Ref.18.

However, the cellular mechanisms involved in the process of GF and GF-CaP materials applications in osteogenic differentiation of stem cells is remain unclear. Few reports said that the 3D graphene foam and 2D graphene substrate can explore key features on both the genomic and protein stages that are involved in the osteogenic differentiation.[19] It has been found that 2D and 3D graphene scaffolds induce differentiation of stem cells into mature osteoblasts at higher levels than the glass or PS substrates. Bone-related genes and proteins were upregulated on graphene regardless of the use of the osteogenic medium. The high gene expressions of MHY10 and MHY10-V2 on 2D and 3D graphene scaffolds recommend that their physical characteristics may have a significant part in the improved differentiation. In conclusion, it is suggested that both chemical and physical properties of GF-CaP act synergistically while presiding osteoblastic differentiation of stem cells.

There are few more evidences on GF-CaP mechanical properties by Y. Zhao et al[20]. HRTEM revealed that the detailed information about microstructures. From Fig. 20a, it can be seen that graphene nano platelets (GNPs) are located on the grain borders bending and succeeding the shape of grain, where the grain size is about 0.5–1 μm, which is consistent with the observation in FESEM images.[20] A single graphene nano particle with thickness of 5 nm was displayed in Fig. 6b, which could be distinguished easily via its characteristic lattice. Graphene nano particles are exceptionally thin, while easy to agglomerate. Overlay GNPs with thickness of about 20 nm are situated between two grains (Fig. 6c), and the lattices of GNPs and grains could see clearly. Also, the tight bonding between matrix and GNPs was clearly displayed in Fig. 6d, and no noticeable diffusion layer is perceived at the interface. The tight bonding is helpful to transferring load between GFs and the matrix and to establishing the bridging, eventually leading to the enhanced mechanical properties.

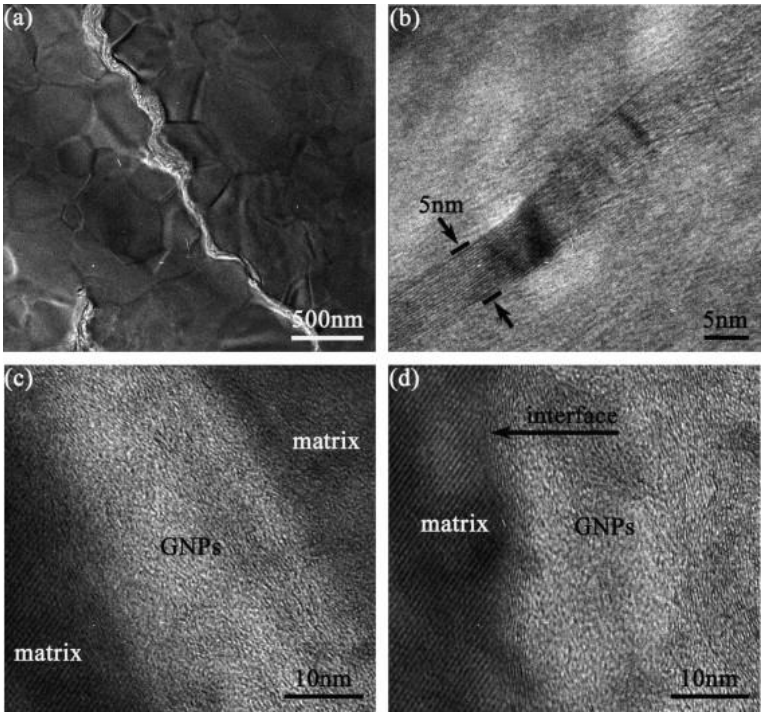

Figure 6. HRTEM images of 1.5 wt% GNPs/BCP composite: (a) GNPs locate at grain boundary, (b) a single GNP with thickness of 5 nm, (c) overlapped GNPs locate between two grains, and (d) the interface of GNPs and matrix. Reproduced with the permission of Elsevier Copyright@2013.Ref.20**.**

This group also proved the high elasticity of the composite with recoverable compressive strain up to 80% (Fig.7a), and significantly improved strength with Young's modulus up to 0.933 MPa compared with that of pure GF template (~7.5 kPa).As shown in Fig. 7b, the original dry and stiff graphene–hydroxyapatite hybrid bioscaffold sample was wetted in culture solvent, and then shifted away with the structure keeping tough and robust. Such superior act of GHBs resisting large

deformation and tension-force-induced shrinkage increases them to be used broadly as biocompatible scaffolds.

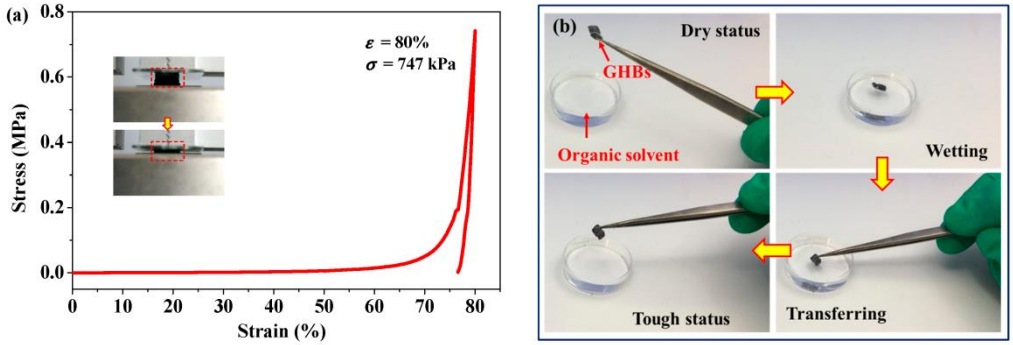

Figure 7. The comparative mechanical compression test of GHB samples with maximum strain up to 80%. Inset is the snapshots during compression. (b) The validation of mechanical robustness of GHB sample during operation in solvent conditions. Adapted from ref 21.

Other reports revealed that the rGO/ Hydroxyapatite Nanocomposites could improve the osteogenesis of MC3T3-E1 preosteoblasts and help new bone formation. The combined HAp, rGO synergistically supported and encourages the natural osteo differentiation of MC3T3-E1 cells without any hindering of their cell proliferation.[22] The boosted osteogenesis (Fig. 8A) was validated from the determination of alkaline phosphatase activity, Alizarin red staining (Fig. 8B), Von Kossa staining (Fig.8C)[20], and Immunoblotting effects (Fig. 8D).[23] The surface adsorption of various oxygenated functions presented in the rGO/hydroxyapatite nanocomposites show better cell compatibility and enhanced biofunctionalities for diverse applications. As one of the matrix mineralization markers, the expression of OCN was calculated by an immunoblot analysis (Fig. 8D), which was corroborated that the matrix was developed via extracellular calcium deposition as displayed above. Once completed 21 days of incubation, the expression level of OCN in MC3T3-E1 preosteoblasts was tremendously ($p < 0.05$) improved by rGO/HAp composites (Fig. 8 D$_B$).[23-24]

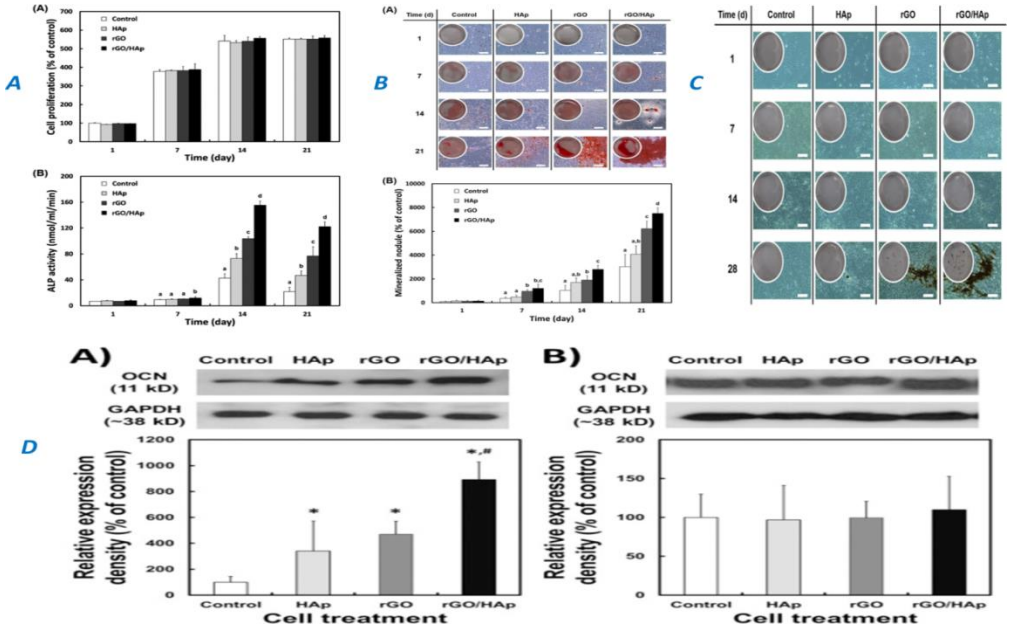

Figure 8. ***A)*** Proliferation and ALP activity of MC3T3-E1 cells incubated with a colloidal dispersion of HAp MPs, rGO NSs or rGO/HAp NCs in BM. (A) During the incubation period (up to 21 d), the presence of rGO/HAp NCs resulted in no appreciable decrease in cell proliferation, compared to the non-treated control. (B) Incubation with rGO/HAp NCs for 7 to 21 d significantly ($p < 0.05$) induced ALP activity. The data is expressed as the mean} SD based on at least duplicate observations from three independent experiments. The different letters in (B) denote the significant difference between the non-treated control and the cells incubated with particles or composites, $p < 0.05$. Ref.20, ***B)***The ARS stain and its corresponding extract in MC3T3-E1 cells incubated with a colloidal dispersion of HAp MPs, rGO NSs or rGO/HAp NCs in BM. (A) Increased calcium deposits by rGO/HAp NCs were not related to the cell number (scale bars = 200 μ m). There was a notable formation of calcium deposits byrGO/HAp NCs from 14 d. (B) The dissolved ARS extracted from the staining plates confirmed that the rGO/HAp NCs significantly ($p < 0.05$) increased extracellular calcium deposition in the cells. The data is expressed as the mean ◉} SD based on at least duplicate observations from three independent experiments. The different letters in (B) denote the significant difference between the non-treated control and cells incubated with particles or composites, $p < 0.05$. All photographs shown in this figure are representative of six independent experiments with similar result. Ref.20.***C)***Image of von Kossa stain in MC3T3-E1 cells incubated with a colloidal dispersion of HAp MPs, rGO NSs or rGO/HAp NCs in BM. Dark brown colored nodular staining was observed at 28 d in cells incubated with rGO/HAp NCs (scale bars = 200 μ m). There was little, if any, crystal formation in cells incubated with HAp MPs alone, whereas the cells incubated with rGO NSs alone exhibited strong positivity for von Koss staining. All photographs shown in this figure are representative of six independent experiments with similar results. Figures adapted from ref.20. ***D)*** Immunoblotting for OCN expression in MC3T3-E1 preosteoblasts treated with rGO/HAp composites in (A) BM and (B) OM. After 21 d of incubation, the expression level of OCN was increased significantly ($p < 0.05$) by rGO/HAp composites. The cells cultured in OM showed no significant difference in the expression level of OCN between the cells treated with and without particles or composites. An asterisk (*) denotes a significant difference between the control and other groups ($p < 0.05$) and number sign (#) denotes a significant difference between the rGO/HAp composite-treated and other groups ($p < 0.05$). The data is expressed as mean ± SD based on at least duplicate observations from three independent experiments. Reproduced with the permission of Elsevier copyrights@ 2015. Ref.23.

3.0 Future aspects and perspectives

GF-CaP are receiving increased interest as a developing presence in material science, chemistry, and biomedical engineering because of their excellent mechanical properties, increased surface area, superior thermal and electronic properties, and simple chemical modifications. Instead of being utilized solely in nanomedicine for phototherapy and drug/gene delivery, GF-CaP exhibits significant interactions and adhesive properties for protein, microbial, and mammalian cells, making GF-CaP hybrid structures promising platforms for various biological purposes. This review summarizes the latest advancements in building GF-CaP structures for biological purposes such as detecting cellular signals, engineering stem cells, coating implants, and regenerating bone tissue. The design of GF-CaPs hybrid architectures is expected to have a promising future and will bring advantages in various research disciplines.

Despite significant efforts to develop GF-CaP-based nano-composites (like 3D printing scaffolds, oriented porous hydrogel, thin film coating), obstacles still remain. Moreover, further investigation is needed to uncover the innate physical and chemical characteristics of biomaterials based on GF-CaPs, as well as to suggest more adjustable methods to create nanostructured film coatings and construct hierarchical porous sprays. Similarly, more potential uses for these hybrid architectures with GF-based CaP coatings should be explored. Numerous applications are still limited to bone regeneration. Recent developments have revealed that the combination of electrical conductivity and mechanical characteristics give GF-CaP-based composites a high potential for use in bone tissue engineering. The large

surface area of GF-CaP coatings allows them to act as carriers that can concentrate growth factors and various types of ECM proteins to support cell adhesion, ultimately promoting the survival and growth of cells in bone research related to stem cell therapy. Different 2D and 3D varieties of GF-CaP hybrid structures have been achieved through methods such as micro/nanofabrication, multilayer coating, free-standing films, and 3D foams. These resources show significant promise for various uses in stem cell and tissue engineering. The exceptional mechanical and electrical characteristics of GF-CaP coatings enable them to control the growth and specialization of stem cells into specific tissues, particularly bones. However, the exact signaling pathways and mechanisms responsible for the differentiation and adhesion of stem cells on GF-CaP based substrates remain unclear. Furthermore, research is required to investigate the possible principles at the cellular/subcellular level and provide fresh evidence for therapies using stem cells. Additionally, it is both fascinating and necessary to explore the interactions between nanostructures and stem cells by incorporating various nanomorphologies, mechanical and electrical stimulations into the design of GF-CaP hybrid substrates, 3D scaffolds, or thin films.

Extensive histological examinations of various organs and tissues are crucial to evaluate potential health hazards associated with non-biodegradable GF-CaP materials before they can be utilized for implantable purposes. Furthermore, various characteristics of graphene-based nanomaterials, including methods for functionalization and bioactivation of calcium phosphate coatings, were thoroughly discussed. Additionally, the discussion touched upon the various impressive qualities of bioactive GF-CaPs and their research opportunities, as well as their potential for development and future prospects. Additionally, the survey questioned the potential of graphene-based scaffold surfaces to serve as stimulating signals for bone cells in order to enhance the bone regeneration process. However, while there is a thorough in vitro analysis of scaffolds, more attention needs to be given to their assessment in vivo, particularly regarding inflammatory reactions, regenerative capacity, and biocompatibility.

Acknowledgments. One of the authors, Associate Professor Dr.Manuri Brahmayya was sincerely grateful to the Professors H.T.Liao and J.P.Chen, CGMH, Taiwan and Administrative office, School of Sciences, Central Tribal University of AP, Vizianagaram, India. MLR institute of Technology, Laxman Reddy Avenue, Dundigal, Hyderabad, Telangana 500043.

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