Chapter 8

The Impact of Graphene Oxide on Bone Regeneration Therapies

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Additional information is available at the end of the chapter

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Abstract

Currently, there are several tissue engineering strategies meant to overcome the incomplete or insufficient bone regeneration conditions offered by autologous bone graft or surgery approaches. In the last decade, attention has been focused toward finding the equilibrium between a suitable scaffold with osteoinductive properties, a cell source with evident potential to develop bone tissue and the appropriate pro-osteogenic factors to condition the differentiation process after cell-scaffold implantation. Consequently, this chapter aims to discuss the benefits that graphene and its derivatives, graphene oxide (GO), bring both to the scaffold biomaterial and to the interaction between the material and the cellular component in order to create a favorable micro-environment for efficient osteogenic differentiation process. Several advantages of including GO in the composition of the materials are shown in relation to cell viability, proliferation, attachment, and osteogenic differentiation.

Keywords: graphene oxide, bone regeneration, cell-scaffold interaction, cell adhesion, cytocompatibility

1. Introduction

New materials with outstanding osteoinductive properties and abilities to promote osteogenesis at the implant site are constantly developed for bone tissue engineering applications. One of these new-generation materials with documented pro-osteogenic effects is graphene [1–3]. Graphene and its derivatives are nanomaterials with specific physical and chemical properties compatible with bone regeneration, and therefore, they possess high potential for bone

tissue engineering approaches. To date, the information about graphene and its derivatives contribution to bone tissue engineering is relatively limited. In this perspective, superior results were reported after graphene functionalization and immobilization of the derivative on different scaffold biomaterials. This approach was successful probably due to the fact that functional groups can reduce the hydrophobic interactions between graphene and the cellular component [4], thus enhancing improved biocompatibility of the resulted material. In particular, graphene oxide (GO) have been promoted as one of the most valuable graphene derivatives with excellent results in bone regeneration [5, 6]. Nowadays, the beneficial effects of graphene and its derivatives are tested in various biomedical applications—anti-cancer therapy, biosensors, drug delivery, and tissue engineering [7–9].

2. GO impact on material bioactivity and cytocompatibility

A very strong interconnection exists between the structural, physicochemical properties, and cytotoxic potential of the materials. Characteristics such as the flat shape, surface charges, and uncontrolled nanobiodegradability of graphene and its derivatives condition a relative nanocytotoxicity that has been reported [10] and currently represents a challenge for the use of graphene-based nanomaterials in clinical applications. Although a lot of positive observations related to the beneficial effects that graphene and GO have on cell growth, expansion, proliferation, and even differentiation of stem cells, caution and safety issues should still be taken into consideration when materials designed with graphene/GO are included in practical tissue engineering.

Most of the *in vitro* studies, which have aimed to evaluate different material compositions with GO content for biocompatibility, have reported a slight decrease in cell viability after contact with GO [11, 12]. However, cell response in contact with biomaterials can vary depending on the GO concentration and the material form of synthesis. Chng and Pumera study from 2013 [13] revealed that GO degree of cytotoxicity was related to the carbon/oxygen (C/O) ratio and the number and distribution of carbonyl residues on the surface of the material. Additionally, the particular conformation adopted by the GO sheets inside a material structure can have an impact on cell behavior in contact to the material [14]. Particularly, a higher degree of compaction in GO sheets determined a lower viability in dermal fibroblasts. This decrease in viability was also associated with the increase in the levels of reactive oxygen species (ROS) in human dermal fibroblasts [14, 15]. Related to this, the activation of caspase-3 pro-apoptotic marker, as well as the release of lactate dehydrogenase (LDH) by PC12 cells, was also reported when the cells were cultured in highly condensed GO sheets materials. These observations lead to the hypothesis that added in very high concentrations to the scaffold or distributed as a very dense network to support material's structure, GO could actually determine a negative influence upon cell viability and response.

For bone tissue engineering purposes, particularly for orthopedic implants, a composite film based on ultrahigh molecular weight polyethylene (UHMWPE) improved with 0.1–1 wt% graphene nanoplatelets was tested for cytocompatibility with bone cells. The cytotoxicity tests

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indicated that the increase in graphene nanoplatelets concentration could decrease bone cells viability over 5 days of culture, possibly due to the agglomeration of particles [16].

Other experiments have shown the contrary—that GO added in certain concentrations in the material has no influence upon cell viability or in some cases even has a positive effect on cell proliferation. In this respect, Sahu et al. [17] has published a study dedicated to thermosensitive hydrogel with GO content in regard to cytotoxicity and concluded that the addition of GO in the composition had no pro-inflammatory effects and that the hydrogel was biocompatible. Studies performed on titanium substrates coated with GO [18] also confirmed that graphene derivatives are biocompatible, present low toxicity, and a large dosage loading capacity, thus being able to function as a carrier for delivery of therapeutic proteins.

Conversely, a series of studies highlighted the importance of functionalizing graphene-based materials in order to minimize its potential cytotoxic effects. Graphene is hydrophobic and easily aggregates in solutions with salts, proteins, ions that can produce toxic effects. Covalent or non-covalent modifications can be performed in order to counteract the cytotoxic-susceptible properties of this material [19]. First, it was observed that the addition of polyethylene glycol (PEG) to GO ensures stability in physiological solutions [20]. Another study [21] emphasized that carboxylated graphene displays higher hydrophilicity and reduced cytotoxicity, due to the fact that carboxylation weakens the hydrophobic interactions between graphene and cellular membranes [19].

Based on positive results reported on grapheme derivates, we have recently tested for cytocompatibility nanomaterials based on polysulfone (PS) and different concentrations of carboxylated graphene (PS/G-COOH). Preliminary observations indicated that cells displayed a very good viability and adhesion in contact with these materials and that proliferation rates were improved as compared with control materials (pure polymer materials) (manuscript under revision).

In the same context, our group published a series of studies highlighting the importance of GO present in either bidimensional (2D) or tridimensional (3D) biomaterials for cell viability and proliferation.

When testing the cytocompatibility of chitosan/GO composite films [22], with 0.5, 1, 2.5, and 6 wt% GO content, MC3T3-E1 murine preosteoblasts adapted faster and proliferated more in contact with the chitosan/GO biocomposites with a higher content of GO. The biocomposite chitosan/GO 6 wt% proved to be biocompatible and displayed the most equilibrated ratio between the pro-proliferative and cytotoxic potential. In this case, viability and proliferation potential was assessed at 2, 4, and 7 days both quantitatively by MTT assay and qualitatively by LiveDead assay and by means of fluorescence microscopy. Fluorescence microscopy images revealed that cells progressively proliferated and reached confluent monolayers on all chitosan/GO biocomposite films, but the cellular density was found to be higher on the composite materials with 2.5 and 6 wt% GO content than that on the chitosan/GO composite films with lower GO content or 2D control. Additionally, a particular cell distribution was noticed for 2.5 and 6 wt% GO biomaterials, suggesting that GO could have an influence on cell behavior and distribution. The composites with 2.5 and 6 wt% GO content registered increased

cell proliferation than the films with low GO loading and controls, particularly after 7 days of culture, as shown by MTT. Conversely, LDH quantification showed a significantly lower profile for chitosan/GO 6 wt% biocomposite than for control chitosan, thus supporting the hypothesis that increase in GO content in material's composition positively influences cell proliferation.

Further on, similar studies were carried out for graphene oxide/chitosan–polyvinyl alcohol films (CS–PVA/GO) in order to determine the cytocompatibility of these materials and the possible interference of GO with cell viability and proliferation [23]. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and X-ray diffraction (XRD) were first employed to assess CS–PVA/GO nanocomposites structural and surface properties. Good GO nanosheets dispersion within the polymer matrix and excellent thermal stability and mechanical strength were shown for these composites, while the highest tensile modulus was obtained for CS–PVA/GO 6 wt%. During biocompatibility tests, an interesting cell distribution was highlighted when the GO concentration increased in the composition of the nanomaterials. Cell alignment and behavior were correlated with the observed GO nanosheets small aggregations within the polymer matrix. Simultaneously, no significant cytotoxic potential was reported for the composites even when increasing the GO concentration to 2.5 or 6 wt% and a general increasing profile of cell viability and proliferation was described during 7 days of *in vitro* culture. Particularly, the composite material with 6 wt% GO proved to display the lowest cytotoxic potential by levels of lactate dehydrogenase released in the cell culture media and to favor most efficiently the proliferation of murine preosteoblasts during 1 week of culture in standard conditions. Statistical significant differences were observed in terms of viability and proliferation between nanomaterials with low GO content (0.5 and 1 wt%) and high GO content (2.5 and 6 wt%).

Similar results were obtained for nanofibrous biocomposite scaffolds of PVA/GO [24] using the same MC3T3-E1 preosteoblasts. In this case, cells were able to grow and attach to the surface of the materials and not change in cell viability was indicated when increasing GO concentration up to 5 wt% in the composition.

A composite with particular good results, holding promises for future biomedical application as a filtration membrane, nanocarrier, or support for bone regeneration, is a bidimensional film based on polysulfone (PS) and GO nanosheets [25]. In this case, PS composites with 0.25, 0.5, and 1 wt% GO were compared in terms of cytocompatibility with PS controls. Based on special conditions of synthesis, the GO nanosheets were uniformly distributed within the PS matrix, thus ensuring a more ordered structure, as revealed by XRD analysis. Clear improvement of thermal and mechanical properties of the composites was revealed when GO was added in the matrix. These changes in the structure were correlated with the bioactivity tested for PS/GO nanomaterials. Very low levels of cytotoxicity were detected during 1 week of culture for all compositions, and no relevant increase in LDH levels was found when 0.25–1 wt% GO was added, suggesting that the low cytotoxic potential of the composite was due to the basal cytotoxicity of the PS substrate. Conversely, quantitative data showed a slight increase in cell viability during 7 days of *in vitro* culture, but statistically significant values were obtained only for the composite with 1 wt% GO, when comparing cell viabilities at 7 and

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4 days of culture. Additionally, the tendency of cell grouping was emphasized by fluorescence microscopy only for PS/GO 1 wt%, as compared to the other composites and to the PS membrane [25].

Similarly, membranes based on poly(ε-caprolactone) (PCL) reinforced with GO nanoplatelets revealed good results toward use in bone regeneration due to the improvements in bioactivity [26]. PCL/GO nanocomposites showed better mechanical properties than PCL films due to the fiber organization and strengthening offered by GO, reflected also in better bioactivity due to the anionic functional groups on GO surface.

Due to the tridimensional structure of the bone, in certain bone reconstruction applications, a tridimensional porous scaffold is required to mimic bone and to resemble the appropriate conditions for regeneration. Thus, tridimensional materials with mechanical and physical–structural properties close to bone were investigated for biocompatibility and potential for bone tissue engineering. In this respect, the cytocompatibility of chitosan/GO scaffolds improved with 0.5 and 3 wt% GO has been tested both by means of indirect and direct studies [27]. Previous reports have shown that chitosan is particularly attractive for bone reconstruction medical applications due to its good biocompatibility, biodegradability, and ability to support osteoblast attachment and proliferation [28, 29]. Remarkably, the addition of GO to the composition of the scaffolds did not affect cell viability, but even resulted in a lower cytotoxicity of the extract collected from chitosan/GO 3 wt% after 24 h of contact with cells. These observations were correlated with the increasing proliferation profile obtained by MTT assay after 7 days of direct contact between murine preosteoblasts from MC-3T3 line and the materials. The data showed that the addition of 3 wt% GO to the chitosan matrix greatly improved the composite properties and bioactivity, suggesting that GO could have positive effects on cell behavior and metabolic activity [27].

Another combination of chitosan (CS) and GO was used as a template to fabricate hydroxyapatite (HA) nanocomposites resembling bone structure [30]. CS–GO–HA and GO–HA matrices displayed good properties to support murine fibroblast and human osteoblast-*like* cells proliferation, but when compared in terms of viability and bioactivity toward mineralization, chitosan functionalized GO matrix provided better conditions for bone repair.

Preliminary positive results for tridimensional GO-containing scaffolds designed specifically for bone tissue repair were also recently reported for gelatin–poly(vinyl alcohol) biocomposites reinforced with GO [31]. In this case, the combination between a naturally occurring compound (gelatin), a synthetically derived one (polyvinyl alcohol) and GO resulted in a biocomposite with equilibrated physical–chemical properties and low cytotoxic profile that allowed murine preosteoblasts viability.

Further tests are required to select the most appropriate biocomposites to serve as platforms to study osteogenic differentiation and thus to validate the most promising biomaterials with application in bone regeneration therapies.

3. GO effects on cell adhesion

In general, it has been shown that the addition of GO favors the interaction between a cellular component and a material substrate, thus ensuring a positive effect on cell adhesion. Several studies [32, 33, 1] have demonstrated that bone marrow mesenchymal stem cells (BM-MSCs) developed a fusiform phenotype with multiple elongations and focal adhesion points in contact with graphene derivatives. These observations support the idea that GO favors cytoskeleton development and enhances cell adhesion to the material that contains GO. Experimental conditions used for 3D scaffolds based on chitosan ± GO or nylon ± GO [34, 2, 6] also concluded that osteoblasts or preosteoblasts adhered better in the presence of GO to the substrate materials. The mechanism underlying GO enhancement of cell adhesion has not been elucidated yet, but Kim et al. [35] suggested that the initiation of focal adhesions is in direct correlation with the nanotopography conditioned by GO.

From our experience, GO also induced a positive effect on murine preosteoblasts adhesion to polysulfone/GO biofilms [25]. A more developed F-actin cytoskeleton has been identified in the presence of 3 wt% GO by confocal microscopy, as compared to the cell cytoskeleton observed for pure polysulfone or plysulfone with 0.5–1 wt% GO addition.

To support this hypothesis, a substrate based on collagen and GO was developed and tested together with rat BM-MSCs for bioactivity in terms of cell viability, cell adhesion, and cell differentiation to bone cells [36]. An obvious dependency of F-actin fiber distribution with the GO content in the biomaterial was reported in this case, confirming our observations.

Other studies [37] described an increased cell adhesion when using GO in conjunction with fibronectin and titanium substrates. In this case, adhesion was evaluated by looking at focal adhesion molecules expression and localization. Vinculin was found to be highly active in the central and peripheral contact area of the cells cultivated in contact with fibronectin and GO.

Good adhesion of cells to their substrate is crucial for cellular processes such as survival, growth, and activation of molecular pathways involved in proliferation. In particular, it has been shown several times that adhesion to the material is essential to induce the molecular program underlying osteogenic differentiation and maturation to functional osteoblasts and osteocytes capable to produce bone-specific extracellular matrix.

4. GO benefits for cell differentiation processes

Scaffolds with different GO content have been previously reported as good substrates for osteogenic differentiation and consequently, for bone tissue regeneration therapies. The ability of graphene and GO to improve the characteristics of scaffold materials and to promote mesenchymal stem cells adhesion, proliferation, and differentiation toward osteogenic lineages has been intensely studied and demonstrated [3, 38, 1, 2, 39]. Lee et al. [33] have reported a proportional correlation between GO presence in the substrate material and the degree of cell osteogenic differentiation. Particularly, this study has highlighted the possibility

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that graphene-based substrates behave like concentration platforms for pro-osteogenic induction factors. Nayak et al. [1] have also shown that GO-covered materials accelerated osteogenic differentiation of human mesenchymal stem cells, as compared to the non-GO-treated-substrates. They concluded that the rate of differentiation conditioned by the GO scaffold is comparable to the osteogenic differentiation induced by specific growth factors and inducers in a conditional media.

Great emphasis has been placed on the development of biomaterials that mimic the structure, composition, and properties of endogenous tissue using the biomimetic method [10]. Since the osteogenic process is based on a combination of signals that will promote the nucleation of hydroxyapatite [40–42], it is essential that the bioengineered scaffold has properties that will induce the assembly of bone-*like* apatite, resembling the natural bone [10]. Considering that charged groups can resemble extracellular matrix proteins and induce the mineralization process, functionalization of GO by bioactive molecules such as dopamine and carrageenan [43] or creation of an interface by modification of GO by gelatin [42] resulted in biomimetic mineralization of hydroxyapatite. Correlated to this enhancement in mineralization, higher cell proliferation, adhesion, and osteogenic potential as shown by alkaline phosphatase activity were reported for MC3T3-E1 preosteoblasts cultured in contact with GO–gelatin surface, as compared to the negative controls [42]. Consequently, these observations can further contribute to the development of more efficient cell–scaffold interfaces based on GO properties for successful application in bone surgery.

Although it was confirmed by an increasing number of studies, the molecular mechanism underlying the ability of graphene or GO to induce by itself the osteogenic differentiation process has not yet been elucidated. Xie et al. [44] designed bidimensional and tridimensional graphene-based substrates to comparatively evaluate the crucial molecular events taking place during periodontal ligament stem cells differentiation to bone cells in these substrates. Bone-specific markers such as RUNX2, collagen type I, osteocalcin were found to be upregulated at gene and protein levels of expression in GO substrates, as a proof of differentiation. A combination of physical and chemical properties of graphene act synergistically to control the osteoinductive effect of graphene [44].

Since they did not show significant cytotoxicity during the biocompatibility studies, graphitic nanomaterials based on carbon nanotubes and carboxylated graphenes were evaluated for capacity to stimulate osteogenesis in the perspective of bone regeneration nanomedicine [45]. The study showed that the activation of the osteogenic differentiation program, synthesis of specific bone markers, and mineral deposition was possible for murine preosteoblasts in MC3T3-E1 cells cultivated in contact with these materials.

An interesting approach in order to evaluate the positive effects of GO on cell differentiation to bone was to incorporate GO nanoparticles in the structure of a scaffold designed for bone tissue reconstruction. Hybrid nanoparticles resulted from reduced GO nanosheets and strontium metallic nanoparticles were then incorporated in poly(ε-caprolactone) matrix with the purpose to test the composite for osteoinductive properties [46]. Increased rates of osteoblast proliferation and differentiation were detected for the scaffold containing GO

nanoparticles, as compared to the control, and this bioactivity was associated with the release of strontium ions from the system.

Apart from its positive influence on cell viability and proliferation, functionalized graphene or GO proved also to favor efficient osteogenesis. By coating fibrin on the surface of GO, a novel nanocomposite (FGO) resulted as a potential solution for bone tissue engineering applications. Based on the analysis of bone markers' profile, release of calcium ions and alkaline phosphatase activity registered in osteoblast-*like* cells MG-63 cultivated in contact with this material, FGO was confirmed to have osteoinductive properties and to be a good candidate for medical applications [47]. Following the same trend of functionalized GO, another group of researchers [48] developed a gelatin functionalized GO composite with the purpose to use the surface charged proteins to mimic mineralization of hydroxyapatite and to obtain functional bone tissue and matrix. The gelatin–GO surface allowed bioactivity as cell adhesion and proliferation, and additionally, it promoted the formation of osteoid mineral matrix during murine cells osteogenic differentiation when compared to control glass surfaces.

The success and efficiency in bone regenerative medicine applications greatly depend on the structure and properties of the implantable biomaterials, but also on the source and type of cells used to condition regeneration. In the past few years, attention was focused on the use of adult stem cells that display the capacity to differentiate toward bone lineage. In this respect, mesenchymal stem cells became most widely used for bone replacement therapies since it was observed their preferential tendency to differentiate to osteogenic lineage when exposed to mechanically stiff scaffolds resembling bone tissue structure. One study [49] showed that when including GO flakes in the composition of soft collagen scaffolds, the resulted composite acquired the necessary stiffness and properties to support MSCs differentiation to bone-*like* cells. Moreover, enhanced osteogenesis was found in cells exposed to GO composite conditions as a result of good MSCs adhesion to the substrate.

An enhanced cell adhesion to the scaffold appears to be crucial for an efficient osteogenic differentiation process. Preosteoblasts, which were previously shown to strongly adhere to fibronectin/GO surface (Fn-Tigra) developed on titanium materials by electrodropping [37], were also shown to differentiate to mature osteoblasts able to produce osteocalcin, type I collagen, and calcium during 2 weeks of culture in contact with this substrate.

Bioceramics became very important in the context of bone tissue engineering. A group of researchers [50] designed a β-tricalcium phosphate covered in modified GO (β-TCP-GRA) and studied the interaction between this bioceramics, GO and stem cells, for bone reconstruction. This combination was found favorable for bone production, since the bioceramics significantly enhanced human BM-MSCs proliferation and osteogenic differentiation, as shown by alkaline phosphatase gene expression levels. Successful osteogenesis was also reported in the case of graphene nanogrids, which promoted the differentiation of human mesenchymal stem cells isolated from umbilical cord toward bone cells [51].

Mesenchymal stem cells isolated from goat cultivated on graphene-coated plates were also used as a potential platform for testing osteogenic differentiation in the view of bone tissue engineering [52]. This study emphasized the ability of oxidized graphene alone to induce

osteogenesis process in goat MSCs in the absence of osteogenic inducers, thus proving the osteoinducing character of graphenes.

However, a small number of studies have focused until present on the effect of GO on human adipose derived stem cells (hASCs) osteogenic differentiation in 3D biomaterials designed for bone tissue engineering [53, 35]. hASCs have revealed encouraging results for adipose and cartilage tissue engineering and proved to be a valuable and more accessible source of adult stem cells than MSCs isolated from bone marrow. Thus, we have developed a strategy for *in vitro* differentiating hASCs inside chitosan-based biomaterials improved with 0.5–3 wt% GO for 28 days in order to study (i) the correlation between GO concentration and the degree of osteogenic differentiation; (ii) osteogenic markers gene expression evolution by qPCR; (iii) osteogenic markers protein expression by confocal microscopy; and (iv) accumulation of bone-specific extracellular matrix by histological staining in our experimental conditions (manuscript in preparation). Our results suggested that the degree of differentiation is strongly influenced by the content of GO in the material and that these materials are suitable for bone regeneration therapies.

Another hybrid scaffold between chitosan and GO was used as a template material for biomineralization of hydroxyapatite and tested as a possible material for bone tissue engineering. This combination proved to be beneficial for cellular activity including proliferation and attachment to the HAP–CS–GO system. Additionally, the scaffold allowed osteoblast growth and an increasing rate of mineralization during *in vitro* cell differentiation, confirming our results and the potential of chitosan/GO nanomaterials for bone regenerative therapies [54].

In the idea of creating an experimental platform for the evaluation of graphene properties for bone regeneration, Lu et al. [55] developed a self-supporting graphene hydrogel film (SGH), which proved to be cytocompatible and to allow cell adhesion and proliferation.

Nevertheless, the great potential of graphene and its derivatives for biomedical applications and their positive effects on cell viability, proliferation, adhesion, and osteogenic differentiation process have been already well documented. At this point, the challenge remains to elucidate the molecular pathways, which are active in the interaction between graphene and the cellular component and to explore and maximize the potential of graphene/GO-based biomaterials as platforms for bone repair therapies and tissue engineering.

5. *In vivo* GO effects during bone regeneration therapies

Regeneration of large bone defects requires development of bioactive scaffolds with distinct properties of promoting stem cells osteogenic differentiation and inducing the *in vivo* new bone formation. There are just few studies with graphene-based composite materials, which demonstrated potential to stimulate osteogenesis *in vivo* (**Table 1**).

Material	*In vivo* model	Post-implant analysis	Biological effects	References
Nanocomposites of reduced graphene oxide (rGO) and hydroxyapatite (HAp) (rGO/HAp NCs)	Rabbit calvarial defects	4 weeks	• relative mRNA expression levels of interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) showed no specific inflammatory responses in the HAp grafts and rGO/HAp grafts • relative micro-CT values for new bone formation were 11.68 ± 8.99, 609.30 ± 308.58 and 1157.83 Å ± 224.52 in the control, Hap grafts, and rGO/HAp grafts • new bone density (%)in the control, HAp grafts, and rGO/HAp grafts were 17.66 (±8.81), 26.80 (±8.32), and 52.85 (±12.04), respectively • conclusion: graphene-based composite materials have potentials to stimulate osteogenesis	[56]
GO-coated titanium implants	Mouse calvarial defects	8 weeks	• BMP-2 delivery using GO-coated Ti found out a higher alkaline phosphatase (ALP) activity in bone-forming cells in vitro compared with bare Ti • substance P (SP), which is known to recruit mesenchymal stem cells (MSCs), was co-delivered using Ti or GO-coated Ti to further promote bone formation • GO-coated Ti induced dual delivery of BMP-2 and SP and increased new bone formation on Ti implanted in the mouse calvaria compared with other groups	[57]
Graphene-oxide-modified β-tricalcium phosphate (β-TCP-GRA) bioceramics	Rabbit calvarial defects	2, 4, and 8 weeks	• micro-CT analysis showed significantly increased new bone formation in the β-TCP-GRA group compared with the β-TCP group; • the volume of the newly formed bone (BV/TV ratio) of the β-TCP-GRA group (26.12 ± 4.44% and 44.83 ± 10.82%) was significantly higher compared with control (16.64 ± 4.57% and 30.41 ± 4.10%) at weeks 4 and 8 post-implant; • trabecular number (Tb.N) in the β-TCP-GRA group (0.39 ± 0.065 and 0.63 ± 0.102) was significantly higher compared with control (0.25 ± 0.049 and 0.41 ± 0.05) at weeks 4 and 8 post-implant; • histomorphometrically analysis of the mineralization area expressed as percentage resulting from the fluorochrome labeling with tetracycline (TE) at 2 weeks, alizarin red (AL) • at 4 weeks, and calcein (CA) at 6 weeks after the implantation surgery, showed increased % of	[50]

Material	*In vivo* model	Post-implant analysis	Biological effects	References
			regeneration and mineralization for β-TCP-GRA group compared with control	
Graphene (GO) flakes suspended in fibrin gels (GO/F) for BMP-2 delivery	Mouse calvarial defects	8 weeks	• micro-CT examination and histological analysis with Goldner's trichrome staining showed that the delivery of various doses of BMP-2 using GO/F resulted in significantly greater bone regeneration than that using F without GO; • a half-dose of BMP-2 delivered by GO/F resulted in bone regeneration similar to that resulting from a full dose of BMP-2 delivered by fibrin gel;	[58]
Graphene hydrogel film	Subcutaneous sites of rats		• stimulate osteogenic differentiation of stem cells, without additional inducer and adequate biodegradability	[55]
Calcium silicate (CS) ceramic reinforced with 1.5 wt% graphene plates (GPs)	Rabbit femur condyle defect	1–3 months	• bone–implant contact ratio reached 84.3 ± 7.4% for GPs/CS coating and 79.6 ± 9.4% for CS coating after 3 months implantation	[59]

Table 1. Platforms to study *in vivo* bone regeneration therapies using graphene-based biomaterials.

Up to date, there is a small number of *in vivo* studies investigating the ability of graphene-based nanomaterial platforms to induce and support production of functional *de novo* bone tissue when practical approaches in bone regenerative medicine require it. Although the implications and benefits for patients experiencing bone defects are of great importance, research toward validation of novel bioimplantable materials designed for bone repair advances in small steps due to safety and ethical requirements. Graphene and its derivatives hold great promise for the synthesis of efficient osteoinductive materials and in-depth research looking at the interplay between graphene effects and molecular pathways active in bone formation will contribute to bringing graphene from bioengineering labs to clinical practice.

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