

Carbon nanocomposites for implant dentistry and bone tissue engineering

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3.1 Introduction

The human skeleton is formed of 206 bones which constitute up to 15% of a person's body weight. In fact, bones are strong enough to bear a load of 525 kg/cm³ and are responsible for the entire framework of the body and protection of its internal organs [1]. In addition, bones are responsible for storage of crucial nutrients, minerals, and lipids, and blood cell production. In the oral cavity, bones are crucial not only to support teeth, but play key roles in speech and mastication, too. Thus, any damage caused to the bone disturbs the normal functioning of the human body [1, 2].

Bone may be damaged or lost due to trauma, tumor, or infections. Bone damage or loss can lead to loss of skeletal mobility, promote muscular overload, and leave essential organs unprotected from external impact [3]. In dentistry, bone loss can also lead to loss of tooth support, formation of crestal defects, or a state of maxillary atrophy [4]. One of the most recurrent bone problems in dentistry is periodontitis, an inflammatory condition caused by bacterial infection that can result in bone destruction, tooth loss, risk of heart disease, respiratory diseases, diabetes, and rheumatoid arthritis [5, 6]. In USA, about 6.5% of adults between age 24 and 64 have periodontal disease, and approximately 10.73% of adults between age 40 and 64 have severe periodontal disease [7]. The traditional treatment for this condition relies on disinfection of the contaminated area, mainly by mechanical debridement and surgical interventions that remove plaque through a deep-cleaning method called scaling and root planing, and medications, followed by flap surgery, if necessary. This common surgery involves lifting back the gums and removing the tartar, after which, the gums heal and fit snugly around the tooth. However, this sometimes results in the teeth being exposed without the protection of hard and soft tissues, rendering the area susceptible to the recurrence of plaque deposition [8]. Hence, these interventions may fail to provide an environment that promotes complete bone regeneration in several ways [4]. To overcome such challenging situations related to bone loss, health professionals often make use of bone grafts to restore bone volume and related functions. Grafts have been widely used, but they have several shortcomings. For instance, autografts demand additional surgical procedures and are related to donor site morbidity, while allografts and xenografts may be prone to immune rejection and infection transmission [9, 10]. Artificial bone

substitutes have been constantly developed. However, these materials also present disadvantages. For example, polymeric materials can improve osteogenic differentiation of mesenchymal stem cells (MSCs) [11], but are often unable to support mechanical loads and mechanical stresses [12]. Ceramics can induce the differentiation of stem cells or osteoprogenitor cells into osteoblasts, but they are brittle and difficult to shape [13, 14].

Recently, researchers have focused on tissue engineering-based strategies to promote bone regeneration. Tissue engineering combines scaffolds, cells, and biologically active molecules to restore or improve damaged tissues [15, 16]. Scaffolds are temporary frameworks that provide a microenvironment for cells to proliferate, differentiate, and generate the desired tissue. These scaffolds can be made of natural, synthetic, or composite materials and often create problems because of low mechanical properties, lack of bioactivity, or degradation rates that are incompatible with the formation of new tissues [16].

To overcome these drawbacks, new nanomaterial-based scaffolds with specific architectures are emerging which are close to mimicking the natural bone architecture at the nanoscale level to improve or enhance attachment, proliferation, and differentiation of cells on the scaffold's surface. These nanomaterials could be used as a coating or mixed with the existing scaffold to enhance their properties or promote differentiation of cells toward osteogenic lineage [17]. In this chapter, we focus on nanobased carbonaceous materials to enhance the outcomes of cell-based tissue engineering for bone regeneration. This is of particularly great interest because these nanomaterials present unique physical, mechanical, and electrical properties that can be functionalized at a mass production level. Thus, their use in tissue engineering could be significant in enhancing and mimicking the natural bone environment.

3.1.1 Carbon nanotubes for bone tissue engineering and regeneration

Carbon is the base of organic chemistry. Several carbon-based materials have been described long ago in the literature, and gradually, there has arisen an increased interest in their use for biomedical applications due to their unique physical, mechanical, and chemical properties. From the various types of carbonaceous materials available, carbon nanotubes (CNTs) and graphene have emerged as interesting candidates to improve the properties of metallic implants.

CNTs are cylinders made of carbon, and were first prepared in 1991 in the form of carbon needle-like tubes [18]. The different number of walls (single, double, or multiwalled) provide these tubes with different properties that can be further explored in various biological, technological, and material applications. These nanosized cylinders can have diameters between 0.7 and 2 nm (for single-walled CNTs) or up to a 100 nm outer diameter for multiwalled CNTs. These can be produced through several routes such as arc discharge, laser ablation, and chemical vapor deposition [18–21]. The latter method has become very popular because it produces CNTs with high purity and eases control of the reaction and dimensions (length and diameter) of CNTs produced [19, 21].

CNTs in their various forms have been explored in a number of biomedical fields for drug delivery, ultrasound imaging, biosensors, and other applications. As these materials present unique properties like high thermal ($5 \times 10^3 \text{ W/m/K}$) and electrical conductivities ($>100 \text{ S/cm}^2$), these have been used to improve the physical and biological performance of materials and scaffolds for tissue engineering and regeneration [18, 22–27].

Due to their ultrahigh tensile strength (up to 200 GPa) and modulus of elasticity (up to 1.34 TPa), CNTs have been used to improve the physical and mechanical properties of polymers such as polystyrene (PS), poly-L-lactide (PLLA), and polycaprolactone (PCL), that are used to produce scaffolds for tissue regeneration. For instance, the addition of multiwalled carbon nanotubes to PCL resulted in composites with increased tensile and compressive moduli. The incorporation of 1 and 2 wt% increased the compressive modulus by 38% and 54% compared to the control. Also, the addition of 2 wt% of CNTs increased the tensile modulus from approximately 85 to 100 MPa [28]. Similar enhancements in mechanical properties via the addition of CNTs were observed on polyurethane scaffolds where the addition of 5 wt% of multiwalled CNTs increased by over 200% on the plateau stress of the foam compared to the control [29]. The addition of 1 wt% of multiwalled CNTs to PS resulted in a composite with 42% increase in the elastic modulus and $\sim 25\%$ increase in the break stress compared to the control [30]. Likewise, 2.5 vol% of multiwalled CNTs added to PS increased the modulus from 1.5 to 3.4 GPa [31]. The addition of nanotubes into a collagen-based construct did not increase the static compressive moduli. However, creep/recovery testing showed that a covalently functionalized nanotubes-collagen composite presented improved mechanical stability under continuous loading [32].

The toughening mechanisms observed by the presence of CNTs at the micron scale are typical of crack deflection at the CNTs/matrix interface, crack bridging by CNTs, and CNT pullout on the fracture surface. Nonetheless, the effectiveness of the CNT reinforcement phase depends on the volume fraction and the alignment of nanotubes inside the materials. Notably, interface debonding and sliding can occur in materials, compromising the enhancements in mechanical properties [33].

Furthermore, the potential of CNTs for bone tissue engineering is not limited to enhancements in the physical and mechanical properties of materials; they could also improve and modulate biological responses. In this regard, CNTs have been extensively explored to improve osteoblastic differentiation and bone formation.

Despite important concerns about the biocompatibility of nanomaterials, substrates containing or made of CNTs have been shown to support the attachment and proliferation of several types of cells such as neurons, smooth muscle, and osteoblastic cells [34, 35]. For the latter, osteosarcoma ROS 17/2.8 cells were capable of attaching and proliferating onto CNT-sprayed glass coverslips. At Day 5, cell growth observed on single-walled CNTs covered about 59% of the maximal growth observed on glass alone. Interestingly, the rate of proliferation on CNTs was related to the electrical character of the nanomaterial. Nanomaterials having nonfunctionalized multiwalled CNTs sustained a significantly higher cell growth than functionalized nanomaterials. Despite the different growth rates, cells on single-walled CNTs presented similar morphology to those on glass with the typical tape-like cytoplasmic prolongations

extending from the flat body [35]. Human osteoblastic cells (SaOs-2) cultured on the multiwalled CNT constructs maintained spherical morphology and displayed a disorganized actin cytoskeleton compared to extremely flat, non-reactive, highly ordered, pyrolytic graphite control surfaces. In addition, cells on multiwalled CNTs presented higher metabolic activity compared to control [36]. Interestingly, the adhesion and proliferation capacity of osteoblastic-like cells depend on the type of CNTs they are seeded onto. For example, cells presented faster adhesion on multiwalled than single-walled CNTs, but the surface of the multiwalled CNT sheets hindered the spread of the attached cells. Cell adhesion was significantly increased by precoating the CNT sheets with serum, showing that the material can adsorb serum proteins that facilitate cell adhesion [37].

Despite these exciting results, the cytotoxic character of CNTs cannot be disregarded if the material is endocytosed. However, the intracellular accumulation of CNTs has been shown to induce cell death. Bronchial epithelial cells (BEAS-2B) took up 18% when exposed to culture medium containing 10 $\mu\text{g/mL}$ of multiwalled CNTs. This resulted in increased gene and protein expression of cytokines IL-6 and IL-8 and migration inhibitory factor [38]. Similar toxic effects were observed in alveolar macrophages that had their phagocytic activity impaired when cultured with medium containing multiwalled CNTs [39]. In another study, the viability of human monocyte-derived macrophage decreased when treated with a solution of MWCNTs at a concentration of 2.5 $\mu\text{g/mL}$ [40]. The literature often shows that CNTs films and scaffolds are cyto- and biocompatible if not endocytosed by the cells [35–37, 41].

Despite the safety concerns that are yet to be fully addressed, CNTs have great potential to improve osteoblastic differentiation *in vitro*. Human adipose-derived stem cells cultured on multiwalled CNTs presented higher expression for ALP, *cbfa1*, and *COLIA1* genes and mineral contents compared to the graphite compact used as control. Cells on multiwalled CNTs also presented increased calcium module formation (Fig. 3.1A) stained by alizarin red than graphite compact control (Fig. 3.1B) [42]. Similar effects were observed for multiwalled CNTs that induced higher gene expression of osteonectin, osteopontin, and osteocalcin, as well as higher levels of alkaline phosphatase activity (ALP/DNA) in osteoblast-like SaoS2, compared to graphite compacts (compressed graphite) [43]. Human mesenchymal stem cells (MSCs) cultured on multiwalled CNT arrays presented higher gene expression of osteocalcin, collagen type I, osteopontin, and *RUNX2* compared to cells cultured on regular tissue culture dishes in the presence of osteogenic medium [44]. Similarly, mammalian cementoblasts (OCCM-30) presented an increased gene expression of ALP, osteocalcin, osteopontin, collagen type I, and *RUNX2* when cultured on carboxyl group-functionalized multiwalled CNTs [45]. One of the plausible explanations for this increased cell differentiation when cultured on CNTs may be the ability of the material to absorb proteins. The preconcentration of fetal bovine serum (FBS) on CNTs resulted in increase of ALP/DNA in osteoblast-like SaoS2 by 18 times compared to pressed graphite [46]. Likewise, the presence of covalently functionalized nanotubes allows collagen constructs to bind to fibronectin, a protein capable of enhancing cell adhesion [32].

Besides being used as a substrate to induce cell differentiation, CNTs have the potential to improve osteogenesis, as previously observed with multiwalled CNT

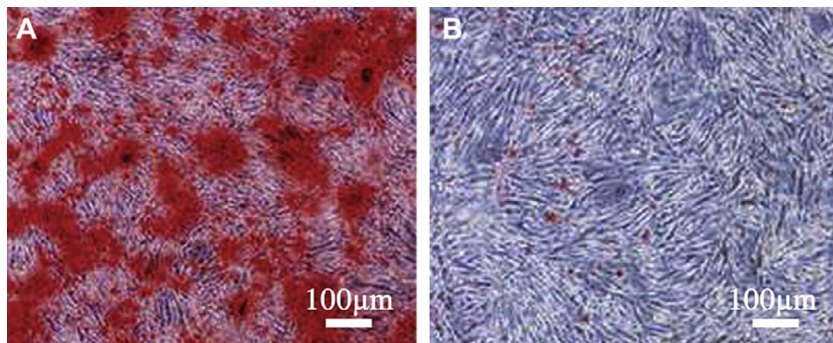


Fig. 3.1 Human adipose-derived MSCs presented increased nodule formation on multiwalled CNTs compared to graphite compacts as shown by the Alizarin red S staining after 7 days with adsorption of rhBMP-2 in advance.

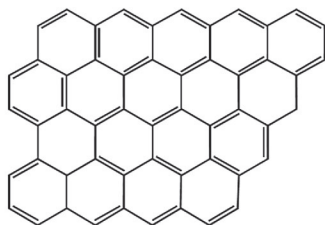
From X. Li, H. Liu, X. Niu, B. Yu, Y. Fan, Q. Feng, et al., The use of carbon nanotubes to induce osteogenic differentiation of human adipose-derived MSCs in vitro and ectopic bone formation in vivo, *Biomaterials* 33(19) (2012) 4818–4827, with permission from Elsevier.

compacts that induce the formation of ectopic bone when implanted in vivo [42]. Hence, CNTs were combined with several biomaterials to improve their potential for bone tissue engineering. For example, hydroxyapatite/vertically aligned multiwalled CNT scaffolds induced biomineralization and apatite deposition in vitro [47]. Also, a composite made of sodium hyaluronate combined with single-walled CNTs increased threefold the trabecula bone formation in sockets after the extraction of mouse molars [48]. The modification of poly(lactide-*co*-glycolide) scaffolds with CNTs increased the attachment, proliferation, and osteogenic differentiation of MC3T3-E1 cells [49]. Similarly, hydroxyapatite/multiwalled CNT composites implanted in the femurs of rats induced the formation of mineralized tissues around the implants. Notably, approximately 55% of the newly formed bone was in contact with the composite [50].

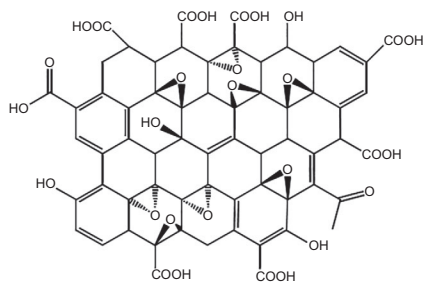
Although the biocompatibility of CNTs still raises concerns, when used either as a substrate or blended with other biomaterials, CNTs can improve and accelerate important processes related to osteogenesis, making this class of materials a promising candidate for bone tissue engineering.

Graphene is another carbon-based material that has received enormous attention recently. It is a two-dimensional single atomic sheet of conjugated sp^2 carbon atoms (Fig. 3.2). It has high thermal conductivity, electron mobility, and is nearly transparent [51–53]. Graphene is very light (0.77 mg/m^2) and presents ultrahigh elastic modulus (up to 2.4 GPa) [53–55]. It is impermeable, even to hydrogen gas, with one of the largest specific surface areas ($2630 \text{ m}^2/\text{g}$) [52, 56, 57]. Graphene can be obtained through several routes. For instance, the micromechanical cleavage of graphite allows the isolation of high-quality graphene flakes, but it is time-consuming and the graphene obtained has area limited to few square micrometers [58, 59]. Alternatively, the CVD relies on pyrolysis of the gases (CH_4 and H_2) and nucleation of carbon atoms on metallic substrates (e.g., nickel and copper) which grow in large domain, producing

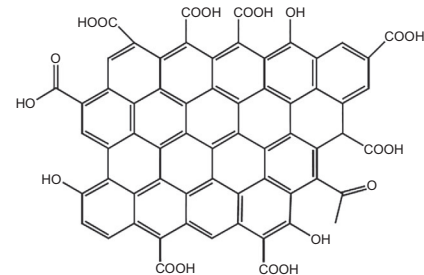
Graphene



Graphene oxide



Reduced graphene oxide



- Mechanical exfoliation
- Chemical exfoliation
- Chemical vapor deposition (CVD)

Chemical treatment of graphite through oxidation, follow by dispersion and exfoliation in water or suitable organic solvent

Thermal or chemical reduction of graphene oxide

Fig. 3.2 Chemical structures of graphene, graphene oxide, and reduced graphene oxide.

From N. Dubey, R. Bentini, I. Islam, T. Cao, A.H. Castro Neto, V. Rosa, Graphene: a versatile carbon-based material for bone tissue engineering, *Stem Cells Int.* 2015 (2015) 804213.

thin films of graphene. This is a scalable method to produce high-quality graphene at large scale [60, 61].

Graphene has two derivatives namely graphene oxide (GO) and reduced graphene oxide (rGO). The first is an oxidized form of graphene with oxygen-containing groups that can be functionalized and dispersed in aqueous solution [62–64]. GO is often produced by the liquid phase exfoliation of graphite [65, 66]. In this method, sheets of graphite are separated into graphene layers upon the insertion of oxygen-containing groups onto them. This method of GO production is less expensive compared to other available methods and is also scalable [62, 66]. GO can be further reduced to rGO with the removal of the oxygen-containing groups and the recovery of a conjugated structure [62, 63].

Although the theory of graphene has been explored since the 1940s [67], the material boomed after its isolation and characterization in 2004 by Geim and Novoselov. They were awarded a Nobel Prize “for groundbreaking experiments regarding the two-dimensional material graphene” in 2010. Since then, graphene and its derivatives have been explored for several biomedical applications such as biosensors, drug delivery, photothermal or photodynamic therapies, and others. The potential of graphene to sustain cell growth and enhance differentiation into several phenotypes (e.g., cardiomyocytes, neurons, osteoblasts) has increased interest in using the material as substrates and scaffolds in tissue engineering.

Like CNTs, the biocompatibility of graphene and its derivatives is yet to be fully understood. One of the main challenges on this matter is the great versatility of these materials which present a wide range of sizes, shapes, and functionalities. These factors influence their interactions with cells and tissues.

Notably, several studies show that graphene and GO are cytocompatible when used as two- and three-dimensional substrates. A wide range of anchorage-dependent cells [bone marrow-derived mesenchymal stem cells (BMSC), dental pulp stem cells (DPSC), periodontal ligament stem cells, pre-osteoblasts] are capable of attaching and proliferating on graphene substrates and scaffolds similar to traditional cell culture substrates [68–74]. Likewise, BMSC and DPSC have been shown to proliferate on GO-based substrates [75, 76]. Controversy arises when the materials were suspended in solutions and used to treat cells. For instance, solutions with high concentration of pristine graphene (50 $\mu\text{g/mL}$) decrease cell viability upon accumulation on the cell membrane and increase levels of oxidative stress [77]. Likewise, PC12 cells experience increased production of reactive oxygen species when cultured in the presence of graphene sheet solution (0.1 $\mu\text{g/mL}$) [78]. Conversely, A549 cells exposed to GO did not show cell uptake [79], and no considerable toxicity was observed after the injection of PEGylated graphene in mice (20 mg/kg) after 90 days [80]. These controversial findings on the biocompatibility of graphene family materials arose from their various shapes, sizes, and chemical characteristics. Hence, it is not possible to form a unified assertion regarding the toxicity of graphene family materials. But it is necessary to determine the hazards that specific graphene-based and modified materials can impose on tissues and living animals and set standards to facilitate their use in clinical work in the near future [81].

Apart from these biosafety concerns, graphene has immense potential to move forward in the field of bone tissue engineering and regeneration. In this regard, the materials can be used either as substrates to enhance cellular differentiation or as additives to improve the mechanical properties and bioactivity of biomaterials.

Graphene can be used as a platform to induce osteogenic differentiation of cells. Several reports have shown that two- and three-dimensional substrates (e.g., films and scaffolds) made of graphene offer cytocompatible surfaces that allow the attachment and proliferation of several types of mammalian cells (Fig. 3.3) [22, 69, 71, 72, 74, 82, 83]. In addition, different forms of graphene have the outstanding ability to induce the spontaneous differentiation of stem cells into the osteoblastic lineage. This has been observed for MSCs seeded onto different materials (e.g., polydimethyl siloxane, polyethylene terephthalate, and glass) coated with CVD-grown graphene without the use of inductors for osteogenic differentiation. Over the course of 28 days, the deposition of calcium nodules on the graphene-coated substrates was higher than those observed in the controls [71–73]. Notably, graphene alone increased the expression of bone-related genes and proteins such as RUNX2, osteocalcin, and osteopontin compared to uncoated substrates [71–73]. Similarly, CVD-grown graphene directly synthesized on nitinol (nickel-titanium alloy) shape memory alloy increased the gene expression of osteocalcin, osteopontin, BMP-2, and RUNX2 [84]. Such behaviors have also been observed in CVD-grown graphene scaffolds that increased the expression of osteoblastic-related markers in MSCs without the use of osteogenic media [68, 72]. Recently, DPSCs have shown to attach and proliferate on a graphene oxide-based substrate (Fig. 3.4A and B). They also showed increased expression of genes related to mineral-secreting cells (e.g., MSX-1 PAX-9, RUNX2, DMP-1 and DSPP) when cultured on GO-based substrates without the use of chemical osteogenic inductors (Fig. 3.4C and D) [76].

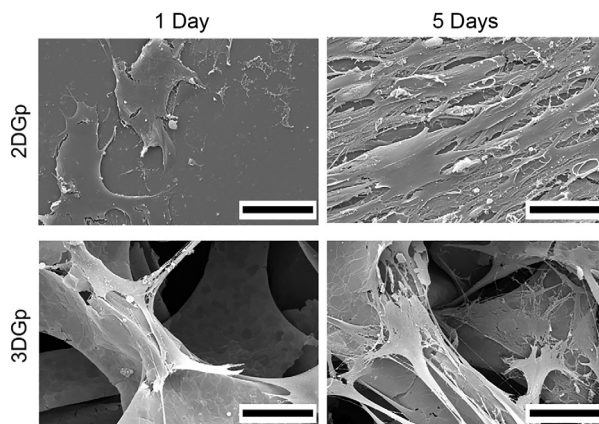


Fig. 3.3 Human periodontal ligament stem cells attachment and proliferation on graphene films (2DGp) or scaffolds (3DGp) for 5 days. Scale = ?.

From H. Xie, T. Cao, J.V. Gomes, A.H. Castro Neto, V. Rosa, Two and three-dimensional graphene substrates to magnify osteogenic differentiation of periodontal ligament stem cells, Carbon 93 (2015) 266–275.

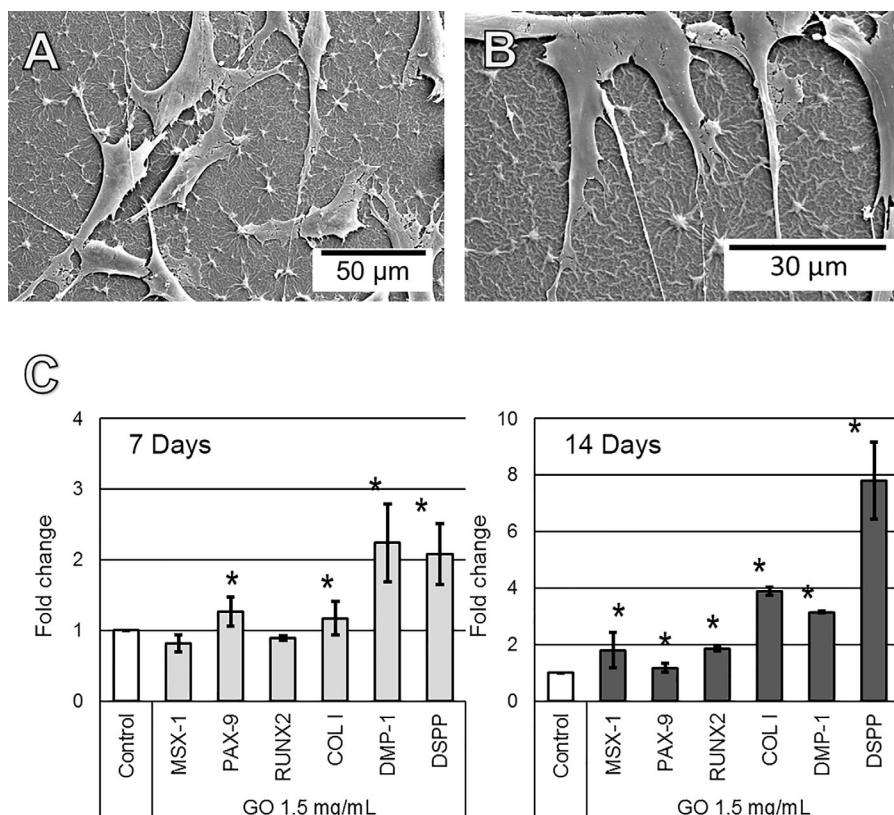


Fig. 3.4 Dental pulp stem cells can attach and proliferate on a graphene oxide-based substrate for 5 days (A and B). After 7 and 14 days, the substrate induced the cells to express markers commonly upregulated in mineral-secreting cells (C). “*” indicate that the expression of the markers is higher than control ($P > 0.05$).

From V. Rosa, H. Xie, N. Dubey, T.T. Madanagopal, S.S. Rajan, J.L.P. Morin, et al., Graphene oxide-based substrate: physical and surface characterization, cytocompatibility and differentiation potential of dental pulp stem cells, *Dent. Mater.* 32(8) (2016) 1019–1025, with permission from Elsevier.

The unstimulated paracrine cells cultured on graphene release soluble factors that promote osteogenic differentiation [73]. It suggests that the physical characteristics of graphene may play a role in the spontaneous osteogenic differentiation observed in MSCs [68, 71–73]. It is known that roughness, elastic modulus, and other physical characteristics of substrates affect stem cell differentiation. DPSCs, when cultured in a rigid hydrogel ($G' = 3600$ Pa), presented higher calcium nodule deposition compared to softer hydrogel ($G' \leq 1570$ Pa) [85]. Graphene has some unique physical properties, such as very high modulus of elasticity (1.0–2.4 TPa [54, 86]), that could trigger mechanosensitive pathways that lead to osteogenic differentiation. In addition, morphological features on the surface of graphene (e.g., wrinkles and ripples) can also influence osteogenic differentiation [71, 87]. These structures can assist the anchorage of cells

and increase the cytoskeletal tension that could induce the spontaneous osteoblastic differentiation [71, 72].

Although graphene's ability to induce spontaneous osteogenic differentiation is exciting, the material boosts differentiation when osteogenic inducers are present. For instance, MSCs seeded on CVD-grown graphene films coated on glass and polydimethyl siloxane in the presence of osteogenic medium, presented higher mineralization potential, and increased gene and protein expression of RUNX2 and osteocalcin, compared to those cells cultured on uncoated substrates [72, 82]. Likewise, GO and rGO substrates have been found to increase osteogenic differentiation in the presence of osteogenic inducers compared to other materials under the same circumstances [82, 88]. This phenomenon is related to the ability of graphene family materials to absorb biomolecules and proteins such as serum, insulin, dexamethasone, β -glycerophosphate, and ascorbic acid [82, 88, 89]. This potentially increases the local availability of important factors, such as dexamethasone that modulates the phosphorylation of RUNX2 or β -glycerophosphate, a source of phosphate for matrix mineralization [90, 91].

The ability of graphene family materials to absorb osteogenic inducers has increased attention on the use of these materials to promote the integration of titanium prosthetic parts and devices into native bone. Titanium is widely used as pins, wires, plates, screws, posts, and implants in orthopedic, plastic/reconstructive, maxillofacial, and general surgery due to its biocompatibility, mechanical properties, and ability to integrate into native bone. However, the integration of titanium parts into native bone takes several months and can be impaired by conditions such as diabetes, osteoporosis, and radiotherapy. This decreases the predictability and increases the probability of failure during treatments [92–95]. In this context, the potential of graphene family materials to induce spontaneous osteoblastic differentiation and their ability to absorb biomolecules and proteins have raised interest in using these materials to improve the osseointegration of titanium. For instance, sodium titanate samples coated with GO (0.1 mg/mL) increased the expression of collagen I, RUNX2, and osteocalcin genes in periodontal ligament stem cells compared to uncoated sodium titanate [96]. Similar enhancement in osteoblastic maturation observed with titanate nanowire scaffolds modified with GO grafted with different chemical moieties ($-\text{COOH}$, $-\text{OH}$, and $-\text{NH}_2$) that increased the proliferation and mineralization potential of MG-63 pre-osteoblasts compared to the unmodified titanate scaffold [97]. Also, dexamethasone-loaded rGO coating deposited on Ti13Nb13Zr enhanced the osteoblastic differentiation of MC3T3-E1 cells by inducing the expression of collagen I, osteocalcin, and osteopontin genes, increasing ALP activity and calcium nodule formation [98]. The capability of GO to bind and release biomolecules and proteins makes this particular form of graphene an interesting platform for the delivery of osteogenic inducers at surgical sites. Both negatively ($-\text{COO}^-$) and positively ($-\text{NH}_3^+$) charged GO can absorb BMP-2, but the release profile depends on the materials' electric character. While positively charged GO releases approximately 65% of all the BMP-2 loaded onto it in the first 24h, the negatively charged version of the material releases only 40% for the same period of time [99]. Notably, GO can release nearly 80% of BMP-2 and substance P attached to it after 14 days [99, 100]. The potential of GO as a carrier for BMP-2 to

improve osteogenesis on titanium implants has been proven *in vivo* when GO loaded with this protein was coated onto titanium samples implanted on the occipital bone of mice. After 8 weeks, the area occupied by new bone was approximately three times higher on the GO/BMP-2-coated titanium compared to the controls (titanium loaded with BMP-2 and titanium alone). In addition, the volume of the newly formed bone was approximately four times more in the presence of the GO-based coatings [100].

Graphene, GO, and rGO can be chemically functionalized and combined with several materials to improve their properties and bioactivity [52–54, 57, 101, 102]. For instance, hydroxyapatite particles have great potential to enhance osteoblastic differentiation but present low fracture toughness and are difficult to shape [103, 104]. These materials can be combined with graphene family materials to improve their physical and mechanical properties in a concentration-dependent manner. Similarly, graphene nanosheets can decrease setting time and increase the hardness of silicate bioceramics [105]. The addition of GO to a hydroxyapatite coating increased the adhesion strength to titanium, and the biocomposite presented higher corrosion resistance compared to the hydroxyapatite alone [106]. Further improvements were observed in fracture toughness, hardness, and elastic modulus of hydroxyapatite when combined rGO or graphene nanosheets [107, 108]. The enhancements observed in bioceramics were not exclusive to hydroxyapatite alone, but were also observed in bioactive glasses [109]. The addition of graphene to 58S bioglass can double the compressive strength and increase the fracture toughness by 38% of the resultant composite [110]. Likewise, 1 wt% rGO used as a reinforcement particle to 45S5 Bioglass increased the fracture toughness from ~ 0.5 to $1.2 \text{ MPa}\cdot\text{m}^{1/2}$ [111].

In summary, graphene and its derivatives hold great potential to drive the field of bone tissue engineering forward. The materials can be used as films or scaffolds to induce and promote cell differentiation toward osteoblastic lineage. They can also be used as reinforcement particles to improve properties of biomaterials.

3.2 Conclusion

Research on these unique carbonaceous smart materials, when used as substrates for tissue engineering, have shown the materials' ability to allow cell attachment, proliferation, and differentiation into osteoblastic lineages. In addition, these materials can be easily functionalized and combined with biomaterials and biomolecules. These modifications can broaden the range of their application and mitigate their toxicity. Additionally, the bioactivity, physical, chemical, and mechanical properties were improved. These materials also support tissue growth *in vivo*, increasing their potential to be used in tissue engineering *in vivo*. Nonetheless, several issues regarding their biodegradability, toxicity, and mechanisms of interaction with living organisms need to be fully addressed. The adverse effects of these materials greatly depend on the variation in shape, size, concentration (in suspension form), and others. As mentioned earlier, the use of graphene and CNTs in tissue engineering has come under the spotlight only during recent years. Although various *in vitro* and *in vivo* toxicology studies have been performed, their results are still controversial. More research regarding

safety, biodistribution, and toxicity is needed to set standards that will allow the usage of these materials for biological applications. Moreover, further research is needed in the areas of understanding the signaling pathways and the mechanism of material-mediated proliferation, differentiation, and toxicity at molecular, cellular, and tissue levels. Though there are still open challenges, recent findings show the potential of graphene and CNTs to be used in tissue engineering field as substrates or scaffolds to improve bone tissue formation.

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