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**CHEMISTRY**

# GRAPHENE DERIVATIVES AND ORGANIC POLYMERS NANOCOMPOSITES FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE

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Carbon-based materials, especially graphene and graphene derivatives, have proved their wide applicability in various fields. In the biomedical arena, graphene is regarded as the most important technological breakthrough, due to its unique and advantageous properties. In this regard, it allows for surface functionalization that further provides the means for covalently and non-covalently conjugating various molecules and macromolecules. However, studies on its biocompatibility and biodegradability properties are somewhat contradictory, so that its combination with other materials is required. Organic polymers are an example of such materials, that have further enhanced the applicability of graphene-based materials in the biomedical field. Specifically, tissue engineering and regenerative medicine have benefitted from these advancements. Accordingly, the aim of this paper is to provide an overview of the main graphene functionalization strategies and graphene-polymer nanocomposites synthesis methods, as well as their most recent applications in nerve, bone, cartilage, and muscle tissue engineering and regeneration.

*Keywords*: graphene, graphene oxide, polymer nanocomposites, tissue engineering

## 1. INTRODUCTION

Owing to their ubiquitous presence in a great variety of applications, carbon-based materials, including single- and multi-walled carbon nanotubes, graphene, and fullerenes, play fundamental roles in our daily lives [1,2]. Since its discovery in 2004, graphene has been standing in the spotlight of materials science as it is regarded as one of the most important technological breakthroughs [1,3–5].

Graphene, a two-dimensional carbon allotrope nanomaterial, is the simplest form of carbon and the strongest and thinnest nanomaterial produced so far [1,6]. Its structure consists of a honeycomb lattice formed by a crystalline single layer of carbon atoms which are bonded by $sp^2$ hybridization, with a C-C bond length of

0.142 nm, while the distance between two adjacent sheets is 0.34 nm [1,4,6–11]. Each carbon atom forms σ bonds with three adjacent carbon atoms, while the remaining p electrons form π bonds perpendicular to the graphene plane with the surrounding atoms [9,10]. The C-C bonds are very strong and, consequently, the structure of graphene is considerably stable, as the application of an external surface results in the deformation and subsequent bending of the atomic surface inside, in order to offset the external force with no rearrangement or misalignment between the carbon atoms [9,12]. While the main form of graphene consists of a monolayer sheet of carbon atoms, it can also exhibit zigzag formation, including nanoribbons or nanostripes, or it can be found as self-supporting, box-shaped, three-dimensional bilayer, gyroid, and aerogel graphene [11]. Graphene can be fabricated through both top-down and bottom-up approaches [7]. Conventional methods generally include mechanical or liquid phase stripping, redox methods, epitaxial growth, orientation epiphysis, chemical vapor deposition, graphitization, electrochemical or thermal exfoliation, and liquid intercalation [9,3].

However, the term "graphene" has been often used for all graphene derivatives (Fig. 1), which include graphene oxide, reduce graphene oxide, porous graphene oxide, single- and multilayered graphene, graphene sheets, and graphene quantum dots [4,6,7,14]. Therefore, "pristine graphene" has risen as a novel term for describing graphene produced without any oxidation or reduction reactions [14]. Among the previously mentioned graphene derivatives, graphene oxide and reduced graphene oxide are the most widely studied materials (Fig. 2). Graphene oxide is obtained through the chemical oxidation of graphene, being described as heavily oxygenated, owing to the presence of a multitude of oxygen-containing functional groups, including hydroxyl, epoxide, carboxyl, and carbonyl groups [15,16]. However, as graphene oxide is characterized by considerable amounts of defects in its crystalline structure, its conductive, mechanical, and optical properties being consequently affected [17]. Therefore, by employing additional reductive treatments, including electrochemical, chemical, thermal, solvothermal, and green reduction techniques, graphene oxide is transformed into reduced graphene oxide, thus partially regaining its graphene-like properties [17–20]. Specifically, in this manner, the oxygen-containing functional groups are partially or completely removed, and the defects in graphene oxide are repaired [18,20].

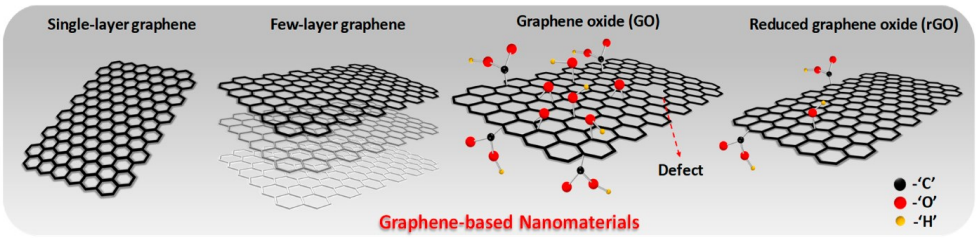


Fig. 1. Various structures of graphene-based nanomaterials.
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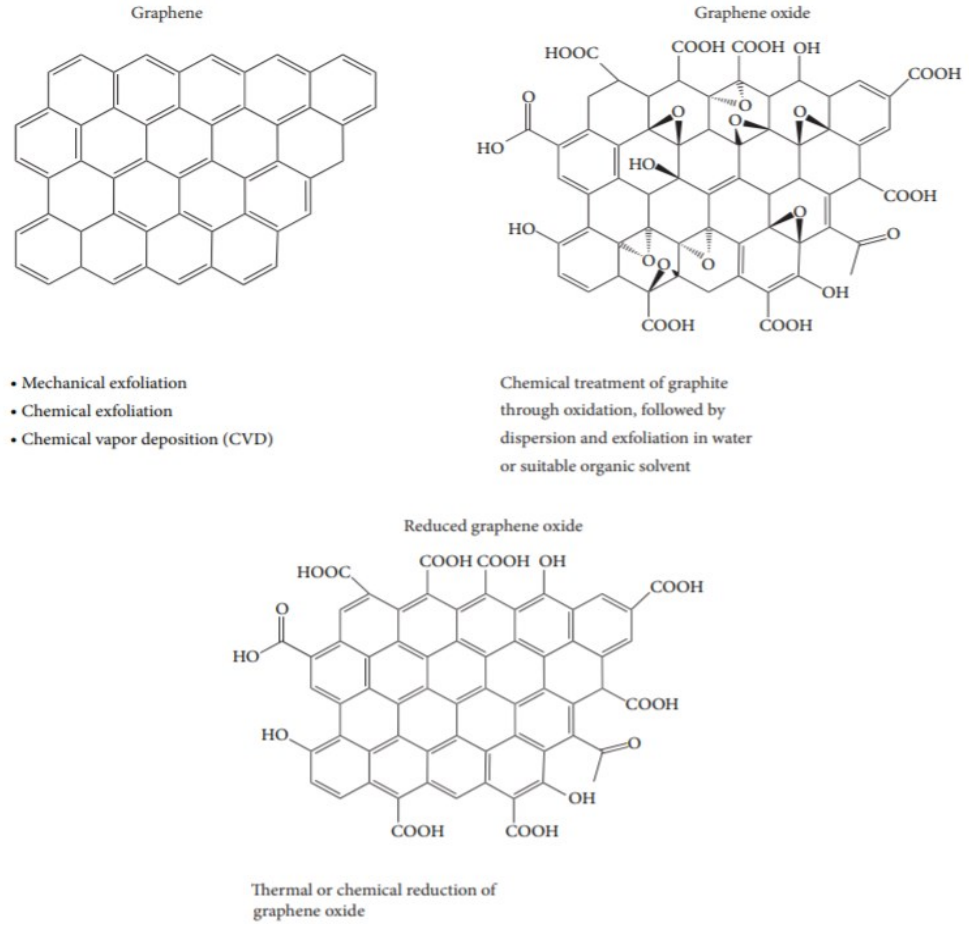


Fig. 2. Main methods for obtaining graphene and graphene derivatives.
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The family of graphene materials present unique and exquisite physicochemical properties, including high surface area, excellent mechanical, electrical, optical, and electrochemical properties, increased thermal conductivity, chemical inertness, and low density (Table 1) [4–10,22–24]. Therefore, it has attracted a great scientific and industrial interest in a wide variety of applications, including (bio)sensing, micro- and nanoelectronics, catalysis, electrochemistry, energy storage, fuel cells, pharmaceuticals, and biomedicine [1,5,7,8,13,22]. Ever since its introduction within the biomedical field in 2008, graphene materials have been intensively studied as drug and gene delivery carriers, bioimaging, biosensing, cell growth platforms, and devices for tissue engineering and regeneration [24–27]. However, publications in the biomedical field regarding graphene biocompatibility are often contradictory [28]. Thus, there are no graphene-based materials successfully employed into real-

patient use [29]. Properties such as morphology, size, and surface modification generally have considerable implications towards cells, tissues, or even the whole body; therefore, the potential toxicity of graphene materials must be carefully investigated and assessed for further clinical tissue engineering applications [2,13,28,29]. Additionally, such applications require certain biodegradability properties, and graphene and carbon-based nanomaterials have been generally assumed to be persistent in the body [30,31].

In this context, their high surface area and rich oxygen and defect networks have allowed for the integration of graphene and graphene derivatives with biopolymers, which has attracted great scientific attention in recent years [8,33]. In this manner, novel nanocomposites with superior properties that can be controlled by adjusting the concentrations of the constituent materials can be obtained [29,34]. Additionally, fabrication of three-dimensional nanocomposite scaffolds with tunable porosities and architectures is possible [29,33]. There is a wide variety of biopolymers that can be used for the synthesis of such nanocomposites, including polyurethane, polystyrene, poly(methyl methacrylate), polyethylene, polycarbonate, polyethylene terephthalate, epoxy, polyaniline, polyvinylidene fluoride, Nafion, and poly (3,4- ethyldioxythiophene), Nylon 6, or polybutylene terephthalate, among many others [35–37].

*Table 1*

Main specific graphene properties [38]

| SPECIFIC SURFACE AREA | BAND GAP | THERMAL CONDUCTIVITY | TRANSPARENCY | CHARGE CARRIER MOBILITY |
|---|---|---|---|---|
| ~2630 $m^2/g$ | 0 | ~5000 Watts per meter-kelvin [W/(m. K)] | ~97.4% | ~200 000 $cm^2/V \cdot s$ |

Therefore, the aim of this paper is to provide an overview of the recent tissue engineering and regenerative medicine applications of nanocomposites comprising graphene or graphene derivatives and organic polymers.

## 1. SYNTHESIS OF GRAPHENE DERIVATIVES AND ORGANIC POLYMERS NANOCOMPOSITES

Graphene and its derivatives are two-dimensional materials widely used as fillers in polymer composites, owing to their excellent resulting properties [39]. However, pristine graphene is generally hydrophobic and has a definite tendency of agglomeration in aqueous solutions and polymer matrices, due to the electrostatic or non-specific interactions between the charged graphene and the environment [40,41]. Therefore, distribution of graphene within the polymer matrix requires additional control at the interface [42]. In this context, the surface of graphene

should be modified or functionalized in order to facilitate its dispersion and stabilization without agglomeration, as well as its solubilization, processability, and interactions with organic polymers [40,41,43]. Surface functionalization of graphene can be performed *via* two main approaches, namely covalent and non-covalent methods (Fig. 3) [41,44,45].

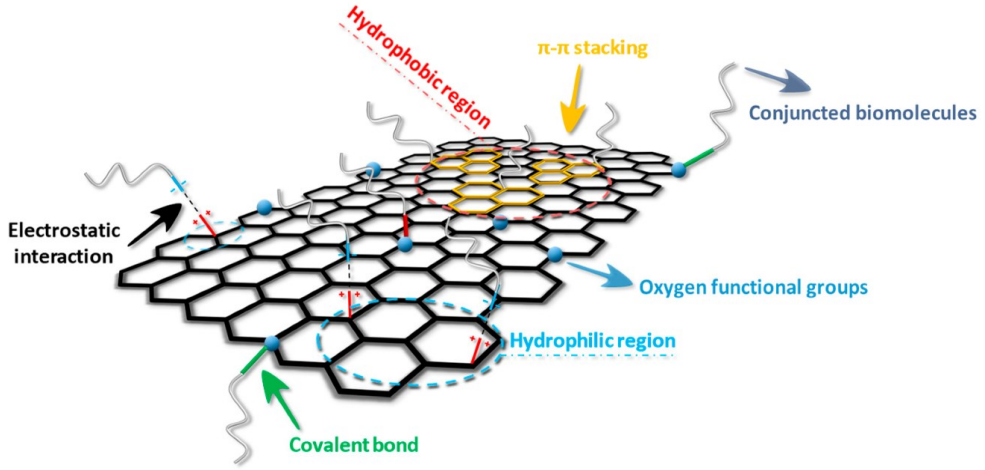


Fig. 3. Schematic representation of the covalent and non-covalent functionalization methods of graphene-based nanomaterials. Reprinted from an open access source [21].

Covalent functionalization methods involve the presence of functional groups bound to the plane structure through strong intermolecular forces, leading to changes of sp2 hybridization and subsequent defect formation and electronic, optical, and mechanical properties loss [43,46]. *The mechanisms responsible for the formation of covalent bonds include the formation of covalent* bonds between free radicals or dienophilic compounds and the C=C bonds within the pristine graphene, and the formation of covalent bonds between organic functional groups and oxygen-containing groups within graphene oxide [41,46]. On one hand, the methods for pristine graphene functionalization are still limited, involving addition of phenyl radicals, diazonium ions, azomethyne ylids, fluorinated phenyl nitrenes, carbenes, perfluorinated alkyl iodides, 2,2,6,6–tetramethylpiperidinyloxy, or benzoyl peroxide [43,47]. On the other hand, the formation of covalent bonds with the oxygen functional groups requires previous oxidation and/ or reduction step through thermal, chemical, or electrochemical methods [44–46], using nitric acid, sulfuric acid, $KMnO_4$, $KClO_3$, or $NaNO_2$ [48,49]. While the presence of oxygen within graphene oxide and reduced graphene oxide has led to tremendous application development, it is not the only useful species for graphene functionalization [40,44,45,50]. In this context, significant work has been performed for the addition of amines, amides, nitro-, thio-compounds, or carbenes or for esterification,

isocyanate modification, or polymer wrapping at the surface of graphene [40,46], through a variety of routes, such as using ionic liquids [40 44,45].

Non-covalent methods involve physical adsorption of the desired moieties onto the surface of graphene through weak interactions, such as π–π stacking or van der Waals, hydrophobic, or electrostatic forces. Non-covalent functionalization generally includes attachment of ionic, metallic, or organometallic compounds, such as $Al^{3+}$, $Mn^{2+}$, $CuO_2^{2-}$, $Fe_3O_4$, or $MnFe_3O_4^{2+}$, or ionic and polymerized ionic liquids, including polyvinylimidazole, polyvinylpyrrolidone, triphenylenes, or pyrene derivatives. Non-covalent methods are highly advantageous in terms of chemical reaction and purification step minimization, and graphene physical properties maintenance. However, as the interactions are considerably weaker than the covalent bonds, functionalization may undergo significant changes in the environment of the targeted application [41,46].

Furthermore, the fabrication of nanocomposites comprising graphene or graphene derivatives and organic polymers can be performed through polymer infiltration methods, in either melt or solution, or through *in situ* polymerization steps [35,39,51]. Additionally, several studies have employed graphene coating onto the surface of the polymeric fibers through wet-, melt, or electrical-spinning [35,48].

Solution mixing or blending is the most straightforward and extensively used method for the fabrication of polymeric nanocomposites [36,52]. Generally, there are three main steps involved in this method. Initially, the graphene material is dispersed and swollen in an appropriate solvent, such as water, chloroform, toluene, acetone, cyclohexane, tetrahydrofuran, or dimethylformamide [36,53,54]. Subsequently, the polymer is introduced and dissolved into the solvent [36,52,55]. In this manner, the polymer chains intercalate and further displace the solvent molecules within the layers of the nanofiller [53,55]. Finally, after proper dispersion of the nanofiller throughout the polymer matrix, the solvent is evaporated, resulting in a nanocomposite structure [36,52–55]. Additionally, nanofiller dispersion often involves the use of energetic agitation, such as magnetic stirring, high-speed shear mixing, reflux, or ultrasonication, in order to separate the nanofiller bundles or agglomerates into a monodispersed state [52–54]. While this method is facile and efficient by ensuring a good dispersion and exfoliation of the nanofiller in the polymeric matrix, the effective solvent removal remains a considerable challenge. Additionally, the high cost and disposal of solvents limit the adaptation of this method for industrial applications. Moreover, the quality of the resulted nanocomposite is dramatically influenced by the quality and quantity of solvent and the time and speed of the mixing reaction [36,54].

Melt processing or blending is the primarily employed technique for industrial manufacturing of polymeric blends, due to its high throughput production, versatility, environmentally friendly nature, and economic suitability [48,56]. It is a cleaner process that involves dispersion of the graphene nanophase

into molten polymers, thus allowing for the development of nanocomposites using insoluble polymers [57,58]. However, the uniform dispersion of the nanofiller into the molten polymer is highly challenging and it generally requires pre-functionalization of graphene [48,57]. Melt processing is often performed through twin-screw extrusion, which is difficult to employ in academic laboratories for research purposes [56,57].

*In situ* polymerization is an efficient method based on the homogenous dispersion of graphene nanophase into a solution containing a neat monomer or multiple monomers, followed by the polymerization of the monomer due to the addition of a suitable initiator, such as heat or radiation [10,37,54,59,60]. This method is highly important for obtaining nanocomposites using insoluble or thermally unstable polymers [54,58]. While most studies demonstrate the formation of covalent bonds between the nanofiller and the polymer matrix, it can also be applied for obtaining non-covalent nanocomposites [10,59]. *In situ* polymerization is the leading technique in terms of uniform and homogenous distribution of the nanofiller throughout the organic matrix [54,61]; however, the increase in viscosity due to the polymerization technique is a considerable disadvantage that limits the manipulation of both blend and load fraction. [10].

Therefore, the properties of graphene-polymer nanocomposites are influenced by the functionalization and synthesis technique, filler dispersity, filler-to-matrix ratio, quality of the filler, solvent and polymer, and filler-matrix bonding [39].

## 3. TISSUE ENGINEERING AND REGENERATIVE MEDICINE APPLICATIONS

The use of various nanostructured fillers for improving the properties of polymer scaffolds has been widely reported. Among them, graphene and its derivatives are some of the most commonly investigated, due to their potential of polymer structural reinforcement, electrical signal generation, and subsequently improved mechanical properties [62]. In this manner, studies have demonstrated the capacity of graphene-polymer nanocomposites to provide a suitable environment for maintaining and promoting cellular proliferation and interactions, thus allowing for the biomimicking of complex natural tissues due to appropriate mechanical and physical cues [25,63]. Therefore, the combination of graphene-based nanomaterials and organic polymers has led to the emergence of a new generation of scaffolds for various tissue engineering applications that could overcome the limitations of the currently available biomaterials [25,62–64]. The most commonly investigated polymers for developing nanocomposites include natural polymers, such as collagen, chitosan, alginate, gelatin, cellulose and silk fibroin, and synthetic polymers, such as polycaprolactone, polylactic acid, poly(lactide-co-glycolide), polyvinyl alcohol and polyacrylamide [65,66].

## NERVOUS TISSUE

Recent advances in the field of tissue engineering and regenerative medicine have allowed for the development of various strategies in nerve tissue repair by using functional scaffolds, such as gels, films, fibers and grooves, that induce neural cell differentiation and proliferation [21]. Additionally, graphene-based materials have proved to stimulate neurogenesis and neural regeneration and regrowth, owing to their excellent electrical conductivity and activity, flexibility, and mechanical strength [21,67–69]. In this manner, electroactive nanocomposites comprising graphene/ graphene derivatives and organic polymers could provide the means for developing functional scaffolds able to both support cellular growth and ensure the synergistic guidance cues required for tissue development [26,68].

In this regard, Gupta *et al*. [70] performed a comparative study between scaffolds based on multiwalled carbon nanotubes and chitosan and graphene nanoplatelets and chitosan for neural tissue engineering. Results showed that graphene-based scaffolds exhibit lower conductivity, but a considerably higher equilibrium constant regarding protein adsorption, thus proving an enhanced potential for cell adhesion. Additionally, graphene reinforcement led to a radial spreading of neurons onto the scaffold, by contrast to carbon nanotube reinforcement, which resulted in an elongated geometry of the neurons. Moreover, another study performed by Jafarkhani *et al*. [71] proved that addition of nano-graphene oxide into chitosan hydrogels led to changes in the pore structure and enhanced mechanical properties, with a consequent increase in nerve cell growth – up to 20%. Furthermore, addition of graphene into silk fibroin, another natural polymer, was also investigated by Zhang *et al*. Thus, incorporation of graphene into electrospun silk fibroin mats resulted in the fabrication of highly conductive scaffolds that promote cell spreading and differentiation and neurite outgrowth by 74.5%. Additionally, the potential of this type of scaffold for tissue engineering applications is also supported by its biodegradability, which allows for tissue growth and replacement [72]. Similarly, addition of graphene into polycaprolactone electrospun fibers improved the mechanical properties of the scaffolds and increased the number of dopaminergic neurons differentiated from rat stem cells [73]. Furthermore, Nezakati *et al*. proved that incorporating graphene flakes into a polyhedral oligomeric silsesquioxane polycaprolactone resulted in major improvements regarding the conductivity and biological properties, namely cell metabolic activity and proliferation, in comparison with the pristine polymer [74]. Moreover, Aval *et al*. developed nanocomposites comprising poly(lactide-co-glycolide)-graphene microribbons, which led to the formation of aligned groove-shaped roughness onto the microribbon surface. Additionally, electrical conductivity, tensile strength, and elastic modulus were considerably improved compared to the poly(lactide-co-glycolide) microribbons. Moreover, results demonstrated an enhanced rate of differentiation of SH-SY5Y cells into mature neurons, thus proving the potential of nanocomposites for central nerve regeneration [75].

Additionally, they also applied graphene-biopolymer nanocomposites for developing bioinks for three-dimensional printing. Specifically, they reported a nano-bioink comprising gelatin methacrylamide, neural stem cells, and graphene nanoplatelets for nerve tissue regeneration purposes. In this manner, the bioink ensures suitable mechanical properties, due to the presence of graphene and biocompatible microenvironments for neural stem cell survival, differentiation, and growth, due to the presence of the natural polymer. The results were sustained by the differentiation of the stem cells into neurons and the elongation of neurites into within the bioprinted construct after two weeks of culturing [76]. Similarly, Huang *et al*. developed a bioink based on graphene, graphene oxide, and polyurethane for neural stem cell bioprinting. Their results proved that the bioink exhibited suitable rheological properties for printing and cell survival. Additionally, addition of graphene-based nanomaterials considerably increased oxygen metabolism and neural differentiation, which proves the potential of these nanocomposites for nervous tissue engineering applications [77].

## BONE AND CARTILAGE TISSUE

While many polymers are continuously developed and improved in order to mimic the structure and functions of the native bone and cartilage tissue, including polylactic acid, polycaprolactone, chitosan, and collagen, their mechanical properties are rather difficult to tune for hard tissue engineering. Therefore, inorganic materials are generally used as reinforcements in polymeric scaffolds [32]. Graphene and its derivatives are an example of such materials, as their ability to promote osteoblast and chondrocyte adhesion and proliferation has been widely proved [38,78]. Their incorporation into polymeric scaffolds reduces the hydrophobic interactions between graphene and cells, thus ensuring an increased biocompatibility of the nanocomposite. Moreover, graphene oxide has proved to be among the most important graphene derivative in bone tissue engineering [79].

Therefore, the literature survey revealed only one study regarding graphene-based polymeric nanocomposites for bone tissue engineering, yet the potential is very high, because addition of graphene and its derivatives induces improved mechanical properties. Specifically, Wang *et al*. investigated the *in vitro* and *in vivo* characteristics of three-dimensionally printed polycaprolactone and graphene scaffolds. As *in vitro* results showed acceptable levels of immune responses, the scaffolds were used for treating calvaria critical size defects in rats, with and without the application of electrical stimulation. As expected, the best results were obtained by the graphene-containing scaffolds with electrical stimulation, as it increased cell migration and influx and, subsequently, novel tissue formation, organization, deposition, and remodeling [80].

By contrast, studies on graphene oxide-based nanocomposites for bone tissue engineering and regeneration are relatively numerous. For example, Purohit *et al*. developed nanocomposite scaffolds comprising gelatin, alginate, and graphene oxide for bone regeneration enhancement. The positive effects of graphene oxide

within the scaffolds were proved by an enhanced compressive strength, overall high swelling capacity, reduced biodegradability, enhanced cell attachment and proliferation of MG-63 cells, and differentiation of mesenchymal stem cells into osteoblasts, thus proving its osteoinductive and osteoconductive properties [81]. Moreover, Dinescu *et al*. investigated the properties, biocompatibility, and bone formation potential of chitosan scaffolds reinforced with 0.5–3% graphene oxide. Results showed that incorporation of the inorganic nanomaterial led to the formation of more ordered morphologies, higher total porosities, and higher surface areas available for the attachment of cells. Additionally, the composite scaffolds allowed for the *in vitro* osteogenic differentiation of human adipose-derived stem cells for 28 days, and *in vivo* bone repair in mice for 18 weeks, with the highest levels of osteogenesis obtained for the 3% graphene oxide-containing scaffolds [82]. In this context, Firoozabady *et al*. also added polycaprolactone to the nanocomposite scaffolds and increased the concentration of graphene oxide to 4%. Results showed that the best morphology, biocompatibility, and biological properties were obtained for the highest concentration of graphene oxide, as it increases the hydrophilicity of the scaffolds [83]. Furthermore, Farshid *et al*. investigated the mechanical properties and the *in vitro* cytotoxicity of single- and multi-walled graphene oxide nanoribbons and nanoplatelets incorporated into highly porous poly(propylene fumarate) nanocomposites. Results showed that the addition of nanomaterials did not induce cytotoxicity in comparison with the polymeric scaffolds and the best compressive properties were obtained for multi-walled graphene oxide nanoribbons, which were higher than the single- and multi-walled carbon nanotubes used as positive controls [84].

For cartilage tissue engineering, Shamekhi *et al*. performed a study on chitosan-based scaffolds reinforced with graphene oxide nanoparticles of different concentrations. Results showed that, by increasing graphene oxide concentration from 0 to 0.1, 0.2, and 0.3%, the mechanical and physical properties of the scaffolds are considerably improved. Moreover, seeding human articular chondrocytes onto the scaffolds leads to enhanced proliferations, especially after 14 days of culturing, as cells revealed more spherical morphologies after 21 days *in vitro* [78]. Additionally, incorporation of graphene oxide into photocrosslinkable alginate conjugated with chondroitin sulfate and gelatin as nanocomposite hydrogel inks to improve shape fidelity and resolution of the printed scaffolds was investigated by Olate-Moya *et al*. Results showed higher cell proliferation of human mesenchymal stem cells from adipose tissue, when compared to pure alginate [85].

## MUSCLE TISSUE

Similarly to nervous cells, myocytes are also capable of undergoing depolarization and repolarization under action potential, which results in their contraction [68]. Therefore, electrically-active nanocomposites could serve as potential biomaterials for enhancing muscle tissue regeneration (Fig. 4).

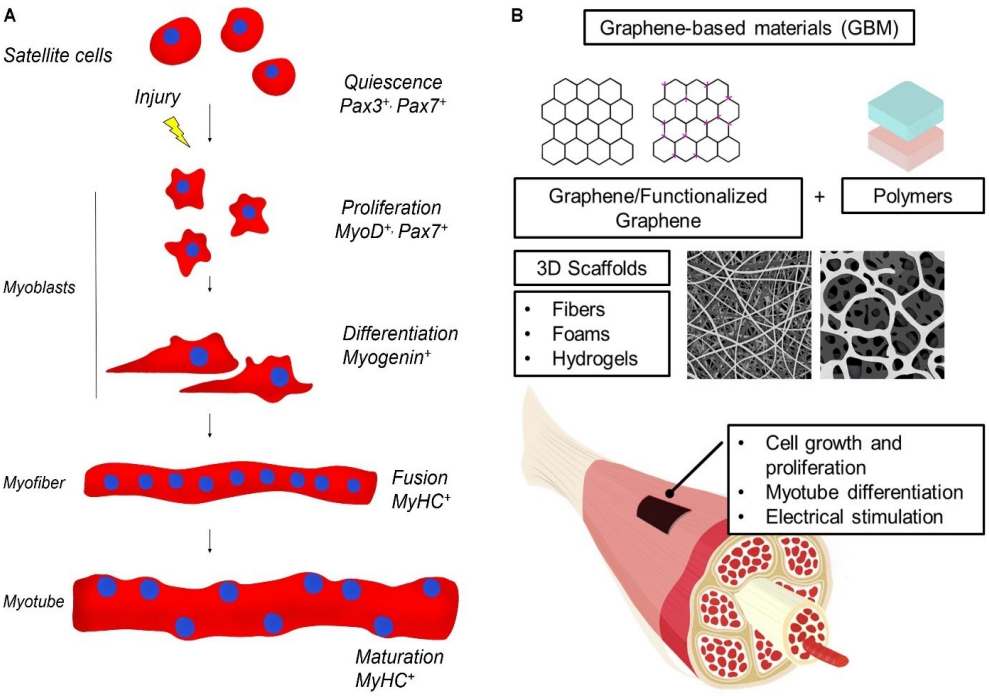


Fig. 4. Schematic representation of skeletal muscle regeneration (A) and the use of graphene-based materials for skeletal muscle engineering (B). Reprinted from an open access source [86].

For now, carbon-based nanomaterials, especially graphene and carbon nanotubes, are two of the most commonly investigated ones in skeletal muscle engineering and regeneration [87]. In this context, Patel *et al*. developed a nanocomposite fibrous hydrogel film comprising graphene, chitosan, and gellan gum. Incorporation of graphene improved hydrogel wettability, electrical conductivity, tensile strength, and toughness, without considerably modifying its elastic properties. Additionally, spreading and myogenesis of mouse myoblast cells seeded onto the nanocomposites were significantly enhanced, as concluded by the formation and unidirectional alignment of multinucleated myotubes [88]. Moreover, Park *et al*. incorporated reduced graphene oxide into polyacrylamide hydrogels through the micropatterning technology. Under electrical stimulation, myoblasts aligned along the lines and subsequently proliferated and differentiated into myotubes, thus serving as an efficient platform for skeletal muscle tissue formation [89]. Furthermore, Jo *et al*. fabricated flexible nanocomposite scaffolds comprising polyurethane and nano-graphene oxide at concentrations of up to 8%. Similarly, incorporation of the carbon-based material enhanced hydrophilicity, elasticity, and stress relaxation capacities of the polymeric scaffolds. Additionally, the C2C12 mouse myoblast cell line seeding showed improved adhesion, spreading, and subsequent proliferation of cells, by up-regulating myogenic mRNA levels and myosin heavy chain expression [90].

## 4. CONCLUSIONS

Graphene and graphene derivatives are highly versatile materials because they allow for the subsequent attachment of various molecules and macromolecules onto their surface. In this manner, their properties can be tuned according to the aim of the application. Functionalization of graphene materials can be performed through both covalent and non-covalent methods. Subsequently, the production of graphene derivatives and organic polymers nanocomposites can be performed through three main techniques, namely solution mixing, melt processing, and *in situ* polymerization. These strategies have allowed for the development of various scaffolds that have been employed in tissue engineering and regenerative medicine applications. Incorporation of graphene-based materials has generally led to increased electrical conductivity, mechanical properties, and cellular proliferation and differentiation. Therefore, it can be concluded that graphene-polymer nanocomposites are potential candidates of great importance in the development of tissue engineering applications. However, the available studies generally lack long-term *in vivo* assessments, which are essential because graphene and its derivatives are synthetic materials and, consequently, the human body can perceive them as a foreign body.

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