

Synthesis, toxicity, biocompatibility, and biomedical applications of graphene and graphene-related materials

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Abstract: Graphene is a two-dimensional atomic crystal, and since its development it has been applied in many novel ways in both research and industry. Graphene possesses unique properties, and it has been used in many applications including sensors, batteries, fuel cells, supercapacitors, transistors, components of high-strength machinery, and display screens in mobile devices. In the past decade, the biomedical applications of graphene have attracted much interest. Graphene has been reported to have antibacterial, antiplatelet, and anticancer activities. Several salient features of graphene make it a potential candidate for biological and biomedical applications. The synthesis, toxicity, biocompatibility, and biomedical applications of graphene are fundamental issues that require thorough investigation in any kind of applications related to human welfare. Therefore, this review addresses the various methods available for the synthesis of graphene, with special reference to biological synthesis, and highlights the biological applications of graphene with a focus on cancer therapy, drug delivery, bio-imaging, and tissue engineering, together with a brief discussion of the challenges and future perspectives of graphene. We hope to provide a comprehensive review of the latest progress in research on graphene, from synthesis to applications.

Keywords: biomedical applications, cancer therapy, drug delivery, graphene, graphene-related materials, tissue engineering, toxicity

Introduction

Graphene, a two-dimensional sheet of sp^2 -hybridized carbon atoms packed into a honeycomb lattice, has recently garnered much attention for its excellent physical and chemical properties.¹ Graphene was first described as monolayer and crystalline graphitic films by the Nobel laureate Andre Geim and Konstantin Novoselov. Following this breakthrough research, researchers around the world have shown much interest in exploring the properties and applications of graphene which has been hailed as a “miracle material”² or a “wonder material”.³ Graphene has good electrical conductivity,⁴ high surface area,⁵ high strength,⁶ good elastic properties,⁷ good thermal conductivity,⁸ ease of functionalization, chemical inertness,⁹ and gas impermeability.¹⁰ Its various applications in the fields of sensors,¹¹ energy storage devices,¹² fuel cells,¹³ and high-strength materials¹⁴ are remarkable. Graphene materials also have applications in the biomedical sector for therapy,^{15–17} diagnosis,^{18–22} and drug delivery,^{23–27} and no other material has comparable properties. Although some progress has been made in diagnosis and drug delivery, the therapeutic applications of graphene remain in their infancy. This difference in the application of graphene in the biological and nonbiological sectors is owed to the toxicity of chemically reduced graphene oxide (GO).^{28–30} Hence, there is an

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urgent need to identify environmentally friendly, and simple approaches to prepare biocompatible graphene materials for biomedical applications.

Although much progress has been made in various areas of graphene research, much work remains to develop applications for human welfare. Graphene and its derivatives show potential toxicity in biological systems ranging from prokaryotes to eukaryotes, depending on dosage and functionalization with various reducing and stabilizing agents. The available literature suggest that the research on graphene has been extensively growing and graphene is exploited for biological applications including bacterial killing, drug delivery, nanocarrier development, cancer therapy, and tissue engineering. However, the issue of potential toxicity arises due to overwhelming application of graphene in biomedical and non-biomedical products where unintended occupational, consumer, and environmental exposures can occur.³¹ The synthesis, toxicity, and applications of graphene and graphene derivatives have been discussed in different areas of research, and these materials have been reviewed recently by several research groups.^{31–41} In this review, we highlight the biological synthesis of graphene from a green chemistry perspective, detail its synthesis using both chemical and biological methods, and discuss its toxicity, biocompatibility, and biomedical applications in detail.

Structure and properties

Strictly speaking, the term graphene refers to a single layer of graphite. More generally, it refers to fewer than ten layers of graphite.⁴² Like carbon nanotubes, fullerene, graphite, and diamond, it is an allotrope of elemental carbon with sp^2 -hybridized carbon atoms, with partially filled p-orbitals above and below the plane of the sheet. It is the thinnest, honeycomb lattice structure composed of carbon atoms.⁴² Graphene materials can be classified in two ways: based on their structure and stacking arrangement. Based on stacking arrangement, they are classified as ABAB type (Bernal stacking), ABCABC type (rhombohedral stacking), and AAA type; Raman imaging is used to determine the stacking arrangement.^{43,44} Graphene with no regular stacking order is classified as “turbostratic”. Stacking of graphene with other materials to form hybrid materials with the desired properties for specific applications has also been attempted. Recently, Hunt et al⁴⁵ reported the stacking of hexagonal boron nitride with a conducting single layer of graphene for band-gap engineering applications to form transistors and other semiconductor devices. Studies have revealed that stacking influences the properties of graphene.^{43,46,47}

Graphene can also be classified based on edge type, as edges also play a key role in determining the properties of the material; graphene materials are divided into those with arm-chair motifs and those with zigzag motifs.^{48,49} Graphene has a carbon–carbon bond length of 0.142 nm,⁴⁴ and it is the thinnest known material with a good strength. The high strength of graphene facilitates its application in flexible electronics and in high-strength composites.^{14,50} Lee et al⁵¹ estimated the Young's modulus values of single- and bi-layer graphene as 2.4 ± 0.4 and 2.0 ± 0.5 TPa, respectively, using Raman spectroscopy. The Young's modulus of graphene has also been determined using molecular dynamics.⁵² The high electrical conductivity is another important feature of graphene.^{53,54} Graphene also has excellent thermal conductivity, gas permeability,^{55,56} and high surface area.^{57–60} The ballistic transport⁵⁴ and the quantum hall effect⁶¹ of graphene are also interesting features that offer immense potential for the applications of graphene-based materials. Unique features such as chemical inertness and ease of functionalization aid in the development of these materials for biomedical applications.⁶² Pure graphene is reported to be biocompatible.⁶³

Synthesis of graphene

Several research groups have thoroughly reviewed the synthesis of graphene.^{64–69} However, the synthesis of graphene using biological systems has not yet been explored. Therefore, this review focuses on summarizing the synthesis of graphene using biological systems. Generally, graphene synthesis is classified in two categories: top-down and bottom-up. The former approach employs exfoliation of a layer of graphene from a graphitic material. The latter approach involves the building up of graphene using carbon-based materials. The bottom-up approach is simple, but it produces material with relatively more defects than the top-down approach. Top-down approaches separate the stacked sheets by disrupting the van der Waals forces that hold the sheets together. Damaging of the sheets during the exfoliation process and reagglomeration of the separated sheets are some of the disadvantages of the top-down technique. The other disadvantage is that graphite, the precursor, is scarce. The bottom-up approach, on the other hand, requires very high temperature. Top-down approaches include micromechanical exfoliation, electrochemical exfoliation, electrochemical and chemical reduction strategy, exfoliation of graphite oxide, solvent-based exfoliation, arc discharge, and unzipping of carbon nanotubes. The bottom-up approaches include epitaxial growth on SiC, chemical vapor deposition, substrate-free method, and carbonization. Micromechanical

exfoliation is a very simple and commonly used technique for investigation of the fundamental properties of graphene. It involves the use of a scotch tape or any other mechanical means to peel layers of graphene from graphite. It is a slow and labor-intensive technique and is not suited for commercial applications.^{70,71} Electrochemical exfoliation is another simple technique for the synthesis of graphene.^{72,73} Acids are used as surfactants in this process, and so this technique may not be ideal for the synthesis of graphene for biomedical applications.^{74–76} In addition, the surfactants are very difficult to remove. The availability of a biocompatible, nontoxic surfactant for use in this technique will greatly aid in developing better methodologies for synthesis. Coupling of sonication with intercalation and solvent-assisted thermal exfoliation has also been reported for the synthesis of GO. Expanded graphite formation, which involves treating graphite with strong acids coupled with sonication or thermal treatment, is another physiochemical option for the synthesis of graphene.⁷⁷ Exfoliation of graphite oxide is another method for the synthesis of graphene.^{78,79}

To date, several methods are available for the synthesis of graphene and its derivatives, including mechanical exfoliation,¹ epitaxial growth,⁸⁰ chemical vapor deposition,^{81,82} unzipping of carbon nanotubes,⁸³ exfoliation of GO,⁸⁴ liquid-phase exfoliation of graphite,^{85–87} ion intercalation and exfoliation,^{88,89} hydro-/solvo-thermal synthesis,⁹⁰ chemical routes,⁹¹ photocatalysis,⁹² photodegradation,⁹³ and electrochemical exfoliation.⁷⁵ All of these methods can produce highly crystalline graphene but are unsuitable for mass production.⁹⁴

Hummer's method is a common method for the oxidation of graphite.⁹⁵ The GO formed is then reduced to form a reduced graphene oxide (rGO). The reduction of GO can be performed with different chemical and biological reducing agents.^{96,97} The most commonly used chemical reducing agents for the reduction of GO are hydrazine,^{98–101} sodium borohydride,^{102–104} Lawesson's reagent,¹⁰⁵ and thiourea.^{106,107} Chemical reduction seems to be a very simple approach; however, it generates a graphene-like film containing low C:O ratio and a considerable quantity of residual functional groups, which leads to highly resistive film.^{108–110}

However, chemical reducing agents are toxic or explosive, resulting in challenges for large-scale production.¹¹¹ The graphene resulting from chemical approach has limited solubility or even undergoes irreversible agglomeration during preparation in water and most organic solvents, unless capping reagents are used, owing to the strong π - π stacking tendency of rGO sheets.^{111–117} The most

commonly used chemical reducing agents are anhydrous hydrazine, hydrazine monohydrate, sodium borohydride, and hydrogen sulfide, which are highly toxic and harmful to living organisms and the environment.^{113,115–117} To enhance the solubility and prevent aggregation problems, several polymers or surfactants have been tested, such as poly(sodium 4-styrenesulfonate), alkaline agents, poly(*N*-vinyl-2-pyrrolidone), poly(allylamine),^{91,118–120} beta-mercaptoethanol,¹²¹ dithiothreitol,¹²² and triethylamine.¹²³

Synthesis of graphene using biomolecules

Biological molecules have been used for synthesis of graphene or reduction of GO due to their easy availability. The synthesis of graphene as a single carbon layer from graphite using thermal, chemical, or electrical treatments is slightly different from that of rGO which is prepared from reduction of GO by chemical or biological methods. The reduction of GO differs based on the reducing agents used, and different reducing agents will produce various C:O ratios and chemical compositions. Recently, usage of biological materials for synthesis of nanoparticles (NPs) has garnered much attention due to their low energy requirements, environmentally friendly nature, dependability, cost-effectiveness, scalability, stability, and availability of the required solutions at high densities, compared with chemical synthesis.¹¹³ Similar approaches have been exploited for the synthesis of graphene using proteins, peptides, bacteria, fungi, plants, and others.

For example, Salas et al¹²⁴ initially proposed "green" reduction of GO via bacterial respiration. Wang et al¹²⁵ demonstrated reduction of GO using respiration of *Shewanella* cells. Interestingly, microbially reduced graphene exhibits excellent electrochemical properties. Subsequently, several laboratories showed synthesis of graphene or reduction of GO using several microorganisms including baker's yeast,¹¹⁴ *Escherichia coli*,^{126,127} *Escherichia fergusonii*,¹²⁸ *Pseudomonas aeruginosa*,¹²¹ *Bacillus marisflavi*,¹¹⁶ and *Ganoderma* spp.¹²⁹ In addition to bacterial systems, several studies have shown usage of plant extracts for reduction of GO. Plants and plant extracts have received much attention for reduction of GO as a suitable alternative to chemical procedures and physical methods.¹³⁰ Extracts from plants may act as both reducing and capping agents in NP synthesis.^{99,131} A few studies have demonstrated reduction of GO using plant extracts including leaf extracts of *Colocasia esculenta* and *Mesua ferrea*,¹³⁰ *Ginkgo biloba* extract,¹²⁹ leaf extracts of cherry, *Magnolia*, *Platanus*, persimmon, pine, maple, and *Ginkgo*,¹³² and extracts of *Pulicaria glutinosa*¹³³ and *Salvadora persica*.¹³⁴ Therefore, plant extracts

could be a potential alternative resource for reducing and stabilizing agents for the synthesis of graphene. A number of studies have also reported GO reduction using various biomolecules, such as ascorbic acid,¹³⁵ amino acids,⁹⁸ glucose,^{136,137} bovine serum albumin,¹³⁸ melatonin,¹³⁹ humanin,¹⁴⁰ enhanced green fluorescent protein,¹¹¹ and resveratrol, a phenolic compound derived from grapes.¹¹³ In particular, use of recombinant proteins for the synthesis of graphene or NPs would save energy and time in downstream processing, and compared to bacterial reduction, this method does not pose the danger of introducing endotoxins.^{141,142} However, most of these procedures limit the use of graphene materials for biological applications. Chemical reduction methods, in addition to polluting the environment, are toxic to prokaryotic/eukaryotic systems. Not only is biological reduction of GO inexpensive, but it also presents fewer burdens, requires less time for reduction process, and most importantly, is nontoxic and biocompatible. Not only is biological reduction of GO inexpensive, but it also presents fewer burdens, requires less time for reduction process, and most importantly, is nontoxic, biocompatible, and high yield. However, one of the challenges posed by biological methods is the purification of graphene from the biomass. Also, they require a number of centrifugation processes. To avoid centrifugation, we can use extracellular-mediated reduction of GO, which is very simple.

Several reports have been published on the detrimental effects of graphene. Schinwald et al¹⁴³ reported the inflammatory effects of graphene in both the lung and the pleural space. However, neither the procedure followed for graphene synthesis nor the functional groups available on the graphene surface were discussed. Whether the graphene molecule alone caused the toxicity or the functional groups formed during synthesis led to toxicity is unclear. In another similar study, the effect of the shape of graphene on cytotoxicity has been reported. Increased lactate dehydrogenase (LDH) release, reactive oxygen species (ROS)-mediated oxidative stress, and caspase 3-mediated apoptosis on exposure to graphene in neural pheochromocytoma-derived PC12 cells have been reported.¹⁴⁴ Thus, it remains unclear whether graphene is safe or toxic.¹⁴⁵

Toxicity of graphene in prokaryotic cells

Nanomaterials are considered to be powerful tools for nanotechnological applications in industry, cosmetics, and health care. In general, NPs appear to be a double-edged sword because they have both beneficial and adverse effects, depending on the context of application. The toxic effects of graphene

can be influenced by physicochemical properties such as size and distribution, surface charge, surface area, layer number, lateral dimensions, surface chemistry, purity, particulate state, surface functional groups, and shape.^{31,146,147} The mechanisms by which GO and rGO nanowalls show toxicity to *E. coli* and *Staphylococcus aureus* bacteria were demonstrated by Akhavan and Ghaderi²⁸ and Hu et al.¹⁴⁸ They demonstrated that both GO and rGO are effective as antibacterial agents. Subsequently, many research laboratories have investigated the potential toxicity of GO and rGO against several bacterial species. For instance, GO and rGO have been reported to enhance bacterial toxicity through enhanced production of ROS in *P. aeruginosa*¹²¹ and *E. coli*,¹²⁷ and cause loss of membrane integrity.¹²⁵ Interestingly, Akhavan and Ghaderi¹²⁶ reported that *E. coli* can reduce GO to bactericidal graphene in a self-limiting manner. Among various types of nanomaterials including graphite, graphite oxide, GO, and rGO, GO showed the strongest antibacterial activity under similar concentrations and incubation conditions, followed by rGO, graphite, and graphite oxide.³⁰ Further, they showed that the antibacterial mechanism included initial cell deposition on graphene-based materials, membrane stress caused by direct contact with sharp nanosheets, and ensuing superoxide anion-independent oxidation. In another report, Liu et al¹⁴⁹ showed that the antibacterial activity of GO sheets toward *E. coli* cells was dependent on the lateral size, time, and concentration. Graphene effectively inhibited the growth of Gram-negative *E. coli* and Gram-positive *Bacillus subtilis* at a concentration of 1 mg/mL.¹⁵⁰ Li et al¹⁵¹ investigated a large-area monolayer graphene film manipulated by charge transfer from a conductor (Cu), a semiconductor (Ge), or an insulator (SiO₂). Graphene films on Cu and Ge inhibited the growth of bacteria by membrane damage and destroying membrane integrity. Figure 1 shows the antibacterial activity of both GO and rGO in *P. aeruginosa* (Gram negative) and *S. aureus* (Gram positive).

Recently, graphene has been used as an antibacterial agent in various nanomaterials. For example, Ma et al¹⁵² reported that silver-modified GO nanosheets (Ag-GO) exhibited superior antibacterial activity toward *E. coli*, due to the synergistic effect of GO and Ag NPs. An Ag-GO nanocomposite displayed high biocidal activity with a minimum inhibitory concentration ranging from 2.5 to 5.0 µg/mL¹⁵³ and better reduction of cell viability than GO.¹⁵⁴ Both Ag NP and Ag-GO samples showed significant antibacterial activity against both Gram-negative and Gram-positive bacteria; in particular, activity was stronger against Gram-negative than against Gram-positive bacteria.¹⁵⁵ Ag NPs anchored on rGO modified with polyethylenimine showed higher antibacterial activity than free Ag NPs.¹⁵⁶

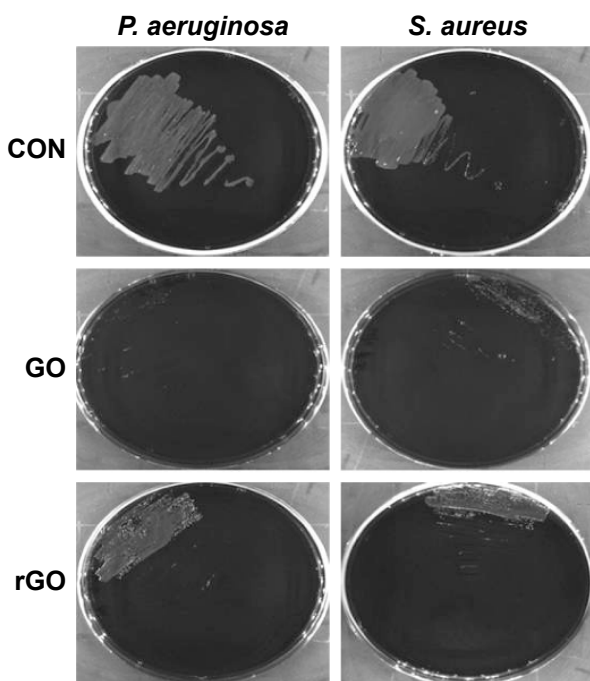


Figure 1 Antibacterial activity of GO and GO reduced by *Evolvulus alsinoides* leaf extract in *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Notes: Cells were incubated with GO and rGO (100 $\mu\text{g/mL}$) separately. Samples were withdrawn at 4 hours and streaked on nutrient agar plates and incubated at 37°C for 24 hours. The differential toxicity of GO and rGO was observed both in Gram-negative and Gram-positive bacteria.

Abbreviations: CON, control; GO, graphene oxide; rGO, reduced graphene oxide.

The functional differences between GO and Ag-GO could be caused by various physical and chemical properties, such as the size and thickness of the sheets, the oxidation ratio, and the solubility.¹⁵³ Ag-GO NPs may cause cell death by the following mechanisms: (i) GO could adhere to or wrap around *E. coli* through hydrogen bonds between the lipopolysaccharides of the bacteria and the oxygenated functional groups of GO.^{107,152,155} (ii) GO could prevent uptake of nutrients from the surroundings while increasing the interaction between Ag NPs and the bacteria,¹⁵² after which Ag NPs favor disruption of the bacterial membrane, leading to inhibition of respiration and replication of bacteria and eventually to cell death.^{153,155–159} (iii) The antibacterial effect of Ag-GO NPs could be caused by the “capturing–killing process”, in which Ag-GO NPs contribute to the deposition of bacteria and increase the contact between the cells and the as-synthesized Ag NPs.¹⁶⁰

In vitro toxicity of graphene in eukaryotic cells

The toxic potential is determined by many factors, among which the interaction between NPs and biological samples is the most crucial.¹⁶¹ In addition, toxicity of graphene in eukaryotic cells depends on several factors, such as chemical

composition, size, surface, shape, use of reducing agents for functionalization of graphene, functional groups, charges, coatings, structural defects of graphene, and dissolving media. Therefore, different studies have reported differing results for NP toxicity. However, general toxicity in eukaryotic cells has been demonstrated. Zhang et al¹⁴⁴ described that the toxic effect of graphene and single-walled carbon nanotubes (SWCNTs) in neural pheochromocytoma-derived PC12 cells was concentration and shape dependent. Interestingly, low concentrations of graphene induced stronger metabolic activity than SWCNTs. LDH levels were found to be significantly increased on exposure to SWCNTs than graphene. Lower concentration of GO has no significant effect on cellular uptake, morphology, viability, mortality, and membrane integrity.¹⁶² Hu et al¹⁶³ reported that the effect of GO was largely attenuated by incubation with 10% fetal bovine serum; the reason was found to be that GO has extremely high protein adsorption ability. Functionalization of graphene by using different reducing agents plays an important role in toxicity. For instance, pristine graphene was shown to cause high oxidative stress by accumulation on the cell membrane, whereas carboxyl-functionalized hydrophilic graphene was not toxic even after internalized by cells.¹⁶⁴ The toxicity of oxidized graphene nanoribbons coated with (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)]) (O-GNRs-PEG-DSPE) was evaluated in four cell lines (HeLa, MCF-7, SKBR3, and NIH3T3) using six different biochemical and cellular assays. The results indicated that O-GNRs-PEG-DSPE have dose- and time-dependent differential cytotoxic effects on the four different cell lines. Among various cell lines, HeLa cells exhibited greater toxicity compared to the other cell lines.¹⁶⁵ However, purified GO showed no significant cytotoxicity in epithelial lung carcinoma cells up to 100 $\mu\text{g/mL}$, and no inflammation or granuloma formation (up to 50 $\mu\text{g/animal}$ dose exposure) in vivo.¹⁶⁶ Lammel et al¹⁶⁷ showed that GO has a dose-dependent toxic effect through plasma membrane damage, that is, loss of plasma membrane structural integrity, which was associated with a strong physical interaction of GO with the phospholipid bilayer. Further, they showed that GO could penetrate the plasma membrane, resulting in altered cell morphology and an augmented number of apoptotic cells. Gurunathan et al¹¹⁶ examined bacterially reduced GO in MCF-7 cells, and found that both GO and bacterially reduced GO exhibit toxicity to MCF-7 cells in a dose-dependent manner. Similarly, GO reduced by extracts of *Ganoderma* spp. showed similar toxicity in MDA-MB-231 human breast cancer cells.¹²⁹ Qu et al¹⁶⁸ demonstrated that

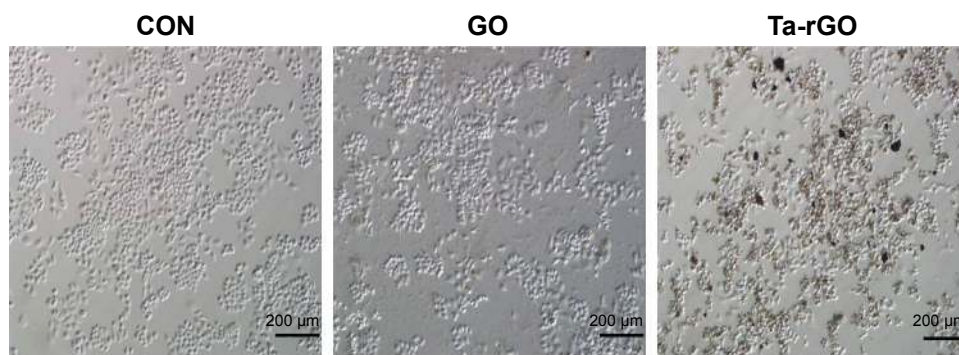


Figure 2 Toxicity of GO and Ta-rGO to human ovarian cancer cells.

Notes: The morphology of human ovarian cancer cells was determined after 24 hours of exposure to GO and Ta-rGO (50 $\mu\text{g/mL}$). Images were captured by interference contrast light microscopy.

Abbreviations: CON, control; GO, graphene oxide; rGO, reduced graphene oxide; Ta-rGO, GO reduced by *Typha angustifolia* leaf extract.

GO induced necrotic cell death in macrophages by activation of toll-like receptor 4 signaling and partly via autocrine production of tumor necrosis factor alpha. Wang et al¹⁶⁹ studied the effects of SWCNTs and GOs using various biochemical assays including cell viability, autophagy induction, and lysosome destabilization in murine peritoneal macrophages, and found that GO molecules were more potent than SWCNTs. Conversely, graphene quantum dots (GQDs) showed low toxicity in HeLa cells.¹⁷⁰ Jaworski et al¹⁷¹ studied the toxicity of both GO and rGO in U87 platelets, U118 glioma cells, and in vivo. The in vitro results indicated that GO and rGO enter glioma cells and show dose-dependent toxicity; rGO was more toxic than GO. In vivo studies suggest that the mass and volume of tumors were reduced after injection of GO and rGO. RAW 264.7 macrophages treated with pristine graphene were found to show increased ROS generation and impairment of mitochondrial membrane potential and cell death by inducing MAP kinases and transforming growth factor-beta-related signaling pathways.^{172,173} Recently, we found that resveratrol-reduced GO induced more toxic effects than GO in human ovarian cancer cells by increasing LDH release, ROS generation, activation of caspase-3, and DNA fragmentation.¹¹³ For example, GO reduced by *Typha angustifolia* leaf extract induced apoptosis by causing morphological changes (Figure 2).

In vivo toxicity of graphene

In vivo toxicity assessment is an essential part of drug delivery research. Graphene could be beneficial or toxic. A biocompatibility study was performed using mice, in which no toxicity was detected in mice exposed intravenously to GO at low (0.1 mg) and medium (0.25 mg) doses, whereas a high dose of GO (0.4 mg) resulted in chronic toxicity.¹²⁵ Another study demonstrated that the functional aspects differed

with size; larger GO particles of 1–5 μm and 110–500 nm accumulated in the lungs, whereas smaller particles were retained by the liver.¹⁷⁴ One study suggested that a 24-hour treatment with nanographene sheets led to accumulation in the reticuloendothelial system (RES) of tumor cells; however, no significant toxicity was observed. In contrast, graphene nanosheets induced pulmonary inflammation, thromboembolism, and immune responses in the lungs of C57BL/6 mice after intravenous administration of 1 mg/kg body weight.¹⁷⁵ On the other hand, nanographene sheets accumulated initially in the RES, liver, and spleen, and later, they were cleared and induced no toxicity at a dose of 20 mg/kg.¹⁷⁶ In another study, various forms of graphene, such as solutions of aggregated graphene, pluronic-dispersed graphene, and GO, were injected directly into the lungs of mice. GO induced mitochondrial generation of ROS, activated inflammatory and apoptotic pathways, and also resulted in severe and persistent lung injury, whereas the mice treated with aggregated graphene and dispersed graphene showed no obvious lung injury.¹⁷⁷ Graphene nanosheets induced cell injury by increasing the levels of various cytokines particularly interleukin-33 and its soluble receptor.¹⁷⁵ A study of the in vivo behavior of dextran-coated GO (GO-DEX) showed that GO-DEX also mainly accumulates in the RES organs at early time points after intravenous injection, and could be gradually excreted over time.¹⁷⁸ Pristine GO induces pulmonary edema and granuloma formation in the lung.^{178,179}

Japanese white rabbits injected intravitreally with GO at concentrations of 0.1, 0.2, or 0.3 mg showed no clinical evidence for ocular changes, and GO had a negligible influence on both the intraocular pressure and eyesight in treated animals.¹⁸⁰ A study in which mice were intravenously injected with polyethylene glycol-treated nanographene oxide (NGO-PEG) at a dose of 20 mg/kg showed that the

NGO-PEG-treated groups at different times postinjection appeared to be normal compared with the control groups and also found no noticeable organ damage or inflammation.¹⁸¹ In addition, short-term exposure to GO-DEX did not induce obvious toxicity in treated animals.¹⁷⁸ The toxicity of graphene depends on surface modifications in vivo. In order to address this issue, Yang et al^{27,33,176} performed time-dependent studies using graphene and PEGylated graphene. One hour after an intravenous injection with 20 mg/kg, polyethylene glycol (PEG)-graphene nanosheets were distributed in many different organs, and after 3 days, PEG-graphene was found mainly in the RES, including the spleen and liver. After 90 days, the PEG-graphene nanosheets produced neither death nor a significant decrease in body weight in the mice. In addition, no significant changes in blood biochemistry or hematology were observed. Furthermore, the liver and kidney functional markers including alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase (ALP) showed no changes. The ratio of albumin and globulin, the urea levels in the blood, and all hematology markers were also unchanged.^{27,33,176} Singh et al¹⁸² found that GO could induce extensive pulmonary thromboembolism in mice. Few-layer graphene with diameters up to 25 μm induced high levels of inflammation in the mouse lung.¹⁴³ The long-term in vivo bio-distribution of intravenously injected nanographene oxide (NGO) functionalized with poly(sodium 4-styrenesulfonate) was systematically examined over 6 months. The evidence from blood biochemistry and histological examinations showed that the NPs mainly accumulated in the lung, liver, and spleen, and caused acute liver injury and chronic inflammation in the accumulated organs.¹⁸³ Zhang et al¹⁸⁴ demonstrated the behavior of mice after short- and long-term administration of rGO. Mice that received a high dose of small or large rGO nanosheets showed little change in exploratory, anxiety-like, or learning and memory behaviors.

Biocompatibility of graphene

Biocompatibility refers to the ability of materials to interact with cells, tissues, or the body without causing harmful effects. Recently, the usage of graphene in stem cell research has been increased due to its unique properties.¹⁸⁵ Liu et al¹⁸⁶ fabricated efficient glucose biosensors through covalent attachment of carboxyl acid groups to GO sheets at the amine residue of GO. The biosensors showed not only good reproducibility and good storage stability but also good adhesion; differentiation of ARPE-19 cells on the GO film was visualized after 72 hours of culture. GO-polyaniline

and graphene-polyaniline hybrid papers showed much higher biocompatibility with the mouse fibroblast cell line L929 than parent papers such as GO or graphene.¹⁸⁷ Park et al¹⁸⁸ fabricated a strong and biocompatible free-standing paper composed of Tween-20 and chemically reduced GO. It showed excellent stability in water and was nontoxic to three mammalian cell lines, Vero cells, embryonic bovine cells, and Crandell-Rees feline kidney cells. Graphene/chitosan hybrid films can repair tissue and improve tissue functions.¹⁸⁹ Graphene conjugated with heparin chains preserved their anticoagulant activity, and showed a much enhanced anti-factor Xa activity of 29.6 IU/mL compared with pristine GO (1.03 IU/mL).¹⁹⁰ Dextran-reduced GO showed significant biocompatibility with HeLa cells, a cervical cancer cell line.¹¹⁵ A study by Lee et al¹⁹¹ demonstrated that graphene- and GO-coated substrates accelerated mesenchymal stem cell (MSC) adhesion, proliferation, and differentiation. Human adenocarcinoma HT-29 cells grown on GO-coated glass slides showed morphological changes and cell enlargement and spreading. Thus, several pieces of evidence support that GO shows promise as a supporting material for cell attachment, growth, and proliferation.¹⁹²

Graphene and GO can support culture of mouse induced pluripotent stem cells (iPSCs) and allows for spontaneous differentiation. iPSCs cultured on a graphene surface exhibited normal cell adhesion and proliferation, whereas iPSCs cultured on a GO surface adhered and proliferated at a faster rate.¹⁹³ Mouse embryonic fibroblast cells treated with microbially reduced GO showed significantly strong viability, and cells grown on plates coated with microbially reduced GO exhibited significant attachment and a higher rate of survival than those treated with hydrazine-reduced GO.¹⁹⁴ Similarly, mouse embryonic fibroblast cells treated with trimethylamine-reduced GO showed increased number and significant attachment.¹²³ GO reduced by spinach leaf extract enhanced ALP activity in mouse embryonic fibroblast cells.¹⁹⁴ Similarly, GO reduced by *G. biloba* extract showed significant biocompatibility and increased ALP activity in human breast cancer cells compared to GO.¹¹⁷ Conversely, Yang et al¹⁹⁵ found that GO could effectively promote dopamine neuron differentiation and further enhancement of dopamine neuron-related gene expression compared with untreated cells. The toxicity or biocompatibility depends on the functionalization of graphene, which can reduce its toxic effects. For example, functionalization of graphene with PEG can minimize oxidation. After 90 days of treatment, histological and hematological analysis showed no considerable toxicity in mice treated with PEGylated graphene

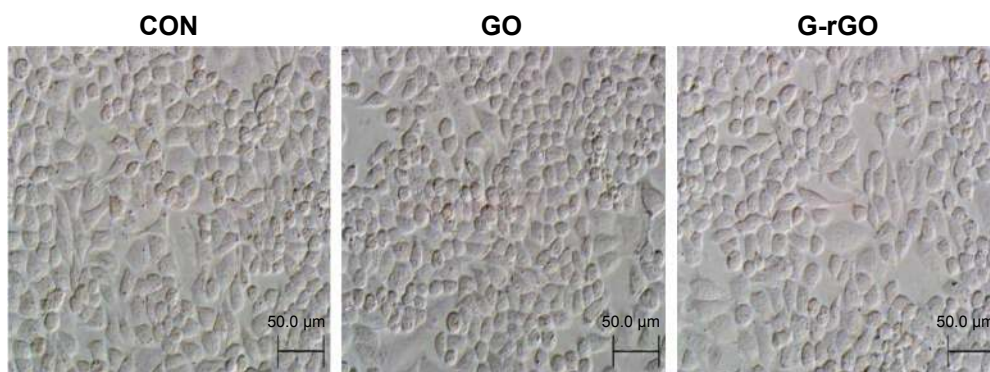


Figure 3 Biocompatibility of GO and G-rGO with human ovarian cancer cells.

Notes: Human breast cancer cells were treated with GO and G-rGO (50 $\mu\text{g}/\text{mL}$) for 24 hours, and then the cells were imaged by light microscopy. Representative microscopic images of GO- and G-rGO-treated cells (50 $\mu\text{g}/\text{mL}$).

Abbreviations: CON, control; G-rGO, glutathione-reduced GO; GO, graphene oxide; rGO, reduced graphene oxide.

(20 mg/kg).¹⁷⁶ Recently, Dubey et al¹⁹⁶ reviewed the detailed role of graphene in bone tissue engineering. Graphene-based materials enhance stem cell attachment and growth for osteogenic differentiation.^{196,197} Several studies have shown that graphene-coated materials are nontoxic and enhance the attachment and proliferation of fibroblasts, osteoblasts, and MSCs.^{191,198–202} Interestingly, Li et al²⁰³ found that graphene can promote neurite sprouting and outgrowth compared to tissue culture plates made of polystyrene. Recently, we found that glutathione-reduced GO showed biocompatibility with human ovarian cancer cells (Figure 3).

Biomedical applications of graphene

Recently, graphene derivatives such as GO and rGO have been shown to exhibit a compatible combination of chemical and physical properties that make them promising candidates for biomedical applications including anticancer therapy, photothermal therapy (PTT), photodynamic therapy (PDT), drug delivery, gene transfection, biosensing and imaging, and tissue engineering.⁴⁰ Cancer is one of the most prevalent diseases globally, and one of the biggest challenges for humanity. Nanotechnology shows excellent promises to attack cancer cells more specifically and effectively, and to reduce undesired side effects.⁴¹ Robinson et al²⁰⁴ developed a photothermal agent using nanosized rGO (nano-rGO) sheets with high near-infrared (NIR) light absorbance and high photothermal efficiency at a low cost. The single-layered nano-rGO sheets were ~ 20 nm in average lateral dimension, containing amphiphilic PEGylated polymer chains and provided stability in biological solutions; they exhibited sixfold higher NIR absorption than non-reduced, covalently PEGylated nanosized GO. Arg-Gly-Asp motif conjugated to nano-rGO enhanced selective cellular

uptake and photoablation in U87MG cancer cells.^{204,205} Folic acid-conjugated GO-loaded nanocarriers with photosensitizers significantly increased the accumulation of chlorin e6 (Ce6) and photodynamic efficacy in tumor cells.^{204,205} Subsequently, Tian et al²⁰⁶ reported that the photosensitizer molecule, Ce6, can be loaded on PEG-functionalized GO via supramolecular π - π stacking. The GO-PEG-Ce6 complex obtained shows excellent water solubility, generation of cytotoxic singlet oxygen, and enhanced intracellular trafficking under light excitation for PDT using photosensitizers. The newly designed GO-PEG-Ce6 complex causes significant cancer cell photodynamic destruction compared to free Ce6. The synergistic effect of chemo-photothermal therapy using doxorubicin (DOX)-loaded NGO-PEG offers higher therapeutic efficacy than chemotherapy or PTT alone.²⁰⁷ A multifunctional nanocomposite named GO-PEG-FA/Gd/DOX-loaded anticancer drug DOX hydrochloride via π - π stacking and hydrophobic interactions exhibited superior tumor targeting and imaging efficiency over free Gd³⁺ and also exhibited a cytotoxic effect in cancer cells.³² GO functionalized with polyethylenimine and polyethylene glycol (GO-PEI-PEG) showed a significant regression in tumor growth and tumor weight after plasmid-based Stat3 siRNA was delivered by GO-PEI-PEG treatment.²⁰⁸ The bioactive molecule, paclitaxel, enhanced by the combination of GO with SWCNTs, caused toxicity to lung cancer cells through activation of ROS and MAP kinase.²⁰⁹ Intravenous administration of protein-assisted fabricated nano-rGO in tumor-bearing mice showed rapid and significant photoacoustic signal enhancement in the tumor region, indicating its excellence for passive targeting and photoacoustic imaging. In addition, the photothermal effect of nano-rGO could efficiently destroy cancer cells.²¹⁰ Reduced graphene oxide

nanomesh functionalized with polyethylene glycol, arginine-glycine-aspartic acid-based peptide, and cyanine 7 (rGONM-PEG-Cy7-RGD) resulted in an ultraefficient PTT (100% tumor elimination 48 hours after intravenous injection of an ultralow concentration [10 $\mu\text{g/mL}$] of rGONM-PEG-Cy7-RGD followed by irradiation with an ultralow laser power [0.1 W/cm^2] for 7 minutes).²¹¹ Yang et al²¹² demonstrated the use of epidermal growth factor receptor antibody-conjugated NGO-PEG containing epirubicin for tumor targeting, and that triple therapeutics showed synergistic effects and also enhanced the local drug concentration (6.3-fold). The combination of triple therapeutic agents significantly suppressed tumor formation and enhanced mouse survival time. Kim et al²¹³ found that DOX-loaded rGO functionalized with polyethylenimine and PEG could escape from endosomes after cellular uptake by photothermally induced endosomal disruption and the proton sponge effect. GO injection not only suppressed tumor progression but also enhanced cell death, autophagy, and immune responses in immunocompetent mice bearing CT26 colon tumors.²¹⁴ Reduced GO nanosheets decorated with mesoporous silica shells have been developed for use in assisted spatiotemporally controlled chemo-photothermal synergistic cancer therapy; they can generate heat under NIR irradiation, and can kill cancer cells very efficiently through the hyperthermia effect.²¹⁵

Drug delivery systems aim to localize delivery of therapeutic agents, in which GO is predominantly used because it can create barrier layers in multilayer thin films, trapping molecules of interest for controlled release.²¹⁶ NGO-PEG has been used as a nanocarrier for delivery of water-insoluble aromatic anticancer drugs into cells. The NGO-PEG loaded with SN38 exhibited high cytotoxicity for HCT-116 cells, 1,000-fold more potent than CPT-11.²¹⁷ Targeted delivery of chemical drugs into cells was achieved using Rituxan (a CD^{20+} antibody) conjugated to NGO-PEG.²⁶ The release of the drug from the GO surface is dependent on pH. Subsequently, Zhang et al²¹⁸ designed folic acid and SO_3H groups conjugated with GO and loaded with DOX and camptothecin via π - π stacking in a controlled manner. GO with a folic acid ligand exhibited specific targeting and enhanced cytotoxicity to MCF-7 cells. Hong et al²¹⁶ fabricated protein-loaded poly-electrolyte multilayer films and demonstrated that proteins can be released in sequence with multiday gaps between the release of each species by incorporating GO layers between protein-loaded layers and found low cytotoxic effect in hematopoietic stem cells. Magnetite NP-decorated reduced graphene oxide (Fe(3)O(4)/rGO) showed successful internalization of Fe(3)O(4)/rGO into the cytoplasm compared to

rGO and significantly higher cytotoxicity in MCF-7 breast cancer cells.²¹⁹ Functionalization of GO with the active targeting ligand TRC105 increased therapeutic efficacy in angiogenesis.²¹⁶ A multiple supramolecular assembly was fabricated with a folic acid-modified beta-cyclodextrin, adamantanyl porphyrin, and GO through non-covalent interactions. Owing to the cooperative contribution of these three units, the DOX showed better drug activity and much lower toxicity.²²⁰ In vitro studies of GO-loaded adriamycin (ADR) showed that GO significantly enhanced the accumulation and toxicity, and reversed ADR resistance compared to free ADR.²²¹ A novel multicomponent graphene nanostructured system containing Fe(3)O(4)(Fe) NPs, PAMAM-G4-NH(2) (G4) dendrimers, and Cy5 on a GO substrate exhibited high dispersion in an aqueous medium, and was magnetically responsive and fluorescent. This system was nontoxic and enhanced the successful uptake and distribution of the GO-G4-Fe-Cy5 nanosystem by MCF-7 breast cancer cells compared to free Cy5.²²² Shen et al³² demonstrated the utility of PEGylated GO for efficient delivery of proteins into cells. In this approach, polyethylene glycol-treated graphene oxide (GO-PEG) delivered proteins to the cytoplasm efficiently, protecting them from enzymatic hydrolysis. Fan et al²²³ developed a water-soluble novel nanocarrier of magnetic Fe(3)O(4) -graphene nanocomposites, which showed excellent dispersibility and stability in aqueous solution and also exhibited superparamagnetic properties. Miao et al²²⁴ fabricated cholesteryl hyaluronic acid-reduced graphene oxide (CHA-rGO) nanosheets that showed increased colloidal stability, safety, and drug-loading capacity in mice. The in vivo antitumor efficacy of DOX delivered by CHA-rGO was significantly increased compared with free DOX or DOX-loaded rGO. Functionalization of GO with VEGF_{121} as the targeting ligand significantly enhanced in vivo tumor vasculature-targeting efficacy and showed excellent in vivo stability.²²⁵

Recently, graphene-based nanocomposites have been used for multimodal bio-imaging and imaging-guided cancer therapy. Graphene and its nanocomposites have emerged as new biomaterials for the development of a new generation of biosensors, nanocarriers, and probes for cell and biological imaging.³² For example, graphene has been proposed as an excellent substrate for biomolecular imaging for introducing nanopores used for DNA sequencing,^{226,227} and as a component in electrodes for neural stimulation.²²⁸ Graphene and graphene derivatives have been used to detect various biological molecules such as dopamine,²²⁹ amino acids,²³⁰ thrombin,²³¹ ATP,²³² and oligonucleotides.²³³ Several specific features

of graphene, including its efficient fluorescence-quenching ability, and its unique electronic properties, have enabled its use in developing biosensors.^{231,232} PEG-modified GO loaded with chemical drugs leverages the intrinsic fluorescence of GO in the NIR region, and gelatin-grafted rGO labeled with a fluorescent dye is used for cellular imaging and drug delivery.^{23,26} Inorganic quantum dots exhibit significantly enhanced fluorescent performance for bio-imaging.^{234–236} In addition, QDs possess unique optical properties such as pH-dependent and upconversion fluorescence behaviors, and QDs are extensively used for cellular imaging in cells, organs, or tissues in this region.^{237,238} Recently, Chen et al²³⁹ reported the synthesis of composites of dextran-coated Fe₃O₄ NPs and GO (Fe₃O₄-GO) as T2-weighted contrast agents for efficient cellular magnetic resonance imaging (MRI). Squaraine dyes were loaded inside mesoporous silica NPs, and the NP surfaces were then wrapped with ultrathin GO sheets; the resulted product exhibited remarkable stability and efficiently protected the loaded dye from nucleophilic attack. This hybrid material is non-cytotoxic and is used for fluorescence imaging.²⁴⁰ Nanographene has been used in noninvasive positron emission tomography imaging for in vivo tumor targeting and quantitative evaluation of drug pharmacokinetics and tumor-targeting efficacy.²¹⁶ Grafted with PEG molecules, GO NPs exhibited high chemical stability at various pH values. Under in vitro conditions, the distribution of GO-PEG NPs in cellular/subcellular components was evaluated using two-photon luminescence imaging. For in vivo imaging, GO-PEG NPs were intravenously injected into mice via the tail vein, and their flow, distribution, and clearance from blood vessels were observed by utilizing a deep-penetrating two-photon imaging technique.²⁴¹ Gollavelli and Ling²⁴² linked a polyacrylic acid bridge with fluorescein *O*-methacrylate to yield multifunctional graphene (MFG) with water dispersibility via a green synthetic approach. An in vitro study of cytotoxicity in HeLa cells revealed that MFG is a biocompatible imaging probe with an IC₅₀ value of ~100 µg/mL; no significant abnormalities or effects on the survival rate were observed after microinjection of MFG. The rGO conjugate, (64)Cu-NOTA-rGO-TRC105, exhibited excellent stability in vitro and in vivo. In vivo, in vitro, and ex vivo studies confirmed the specificity of (64)Cu-NOTA-rGO-TRC105 for tumor vascular CD105.²⁴³ Lalwani et al²⁴⁴ reported that oxidized single- and multi-walled GO nanoribbons exhibit approximately five- to tenfold signal enhancement for photoacoustic tomography in comparison to blood at the wavelength of 755 nm, and ~10%–28% signal enhancement for thermoacoustic tomography (TAT) in comparison to deionized water

at 3 GHz. Oxidized graphene nanoribbons show promise as multimodal photoacoustic tomography and TAT contrast agents, and oxidized graphene NPs are suitable contrast agents for TAT. GO has been coupled with anti-Her2 antibody, which is used for the treatment of breast cancer, and radio-labeled with [In111]-benzyl-diethylene-triaminepenta-acetic acid via π - π stacking for targeted and functional imaging.²⁴⁵ A magnetic graphene complex was used to identify metastatic pancreatic cells in the lymph nodes and also used for direct guided PTT against cancer cells.²⁴⁶ Gollavelli and Ling²⁴⁷ used magnetic graphene as a potential theranostic nanocarrier for MRI and fluorescence dual-modality imaging and for PDT and PTT. Recently, Zhang et al²⁴⁸ developed a new colorimetric assay for the direct detection of cancer cells using graphene as a signal transducer. Interestingly, graphene was used as a biosensor for molecular marker analysis in cancer diagnosis in the field of in vitro detection tool development. Liu et al²⁴⁹ developed a method for reliable quantification of miRNAs in medical research and early clinical diagnostics, which is stable, sensitive, and specific for miRNA detection. The extraordinary fluorescence quenching of GO provided a high signal-to-noise ratio. Due to protection of the target miRNA by GO, cooperative amplification, low background fluorescence, and sensitive and accurate detection of miRNAs have been achieved.

BaGdF5 NPs attached to GO showed low cytotoxicity, positive magnetic resonance contrast effect, and better X-ray attenuation properties than iohexol.²⁵⁰ Yan et al²⁵¹ designed and prepared a novel photo-theranostic agent for enhanced optical imaging using sinoporphyrin sodium-loaded GO-PEG, with improved fluorescence properties. Figure 4 shows the possible application of graphene and graphene-related materials in bio-imaging of live animals.

Tissue engineering uses biological materials for maintenance and improvement of the functions of tissues or organs. Graphene and its derivatives are having a crucial role in tissue engineering due to their unique physicochemical properties. In order to spread, proliferate, and perform their functions, cells or tissues need a good substrate, and graphene and GO can play this vital role in tissue engineering. Due to their mechanical properties, they are suitable for the structural reinforcement of biocompatible films, hydrogels, and other scaffolding materials frequently used in tissue engineering.³¹

MSCs are multipotent progenitor cells derived from adult bone marrow, and have shown promising applications in tissue repair and cell therapies.^{252,253} The differentiation of MSCs is controlled by several factors including

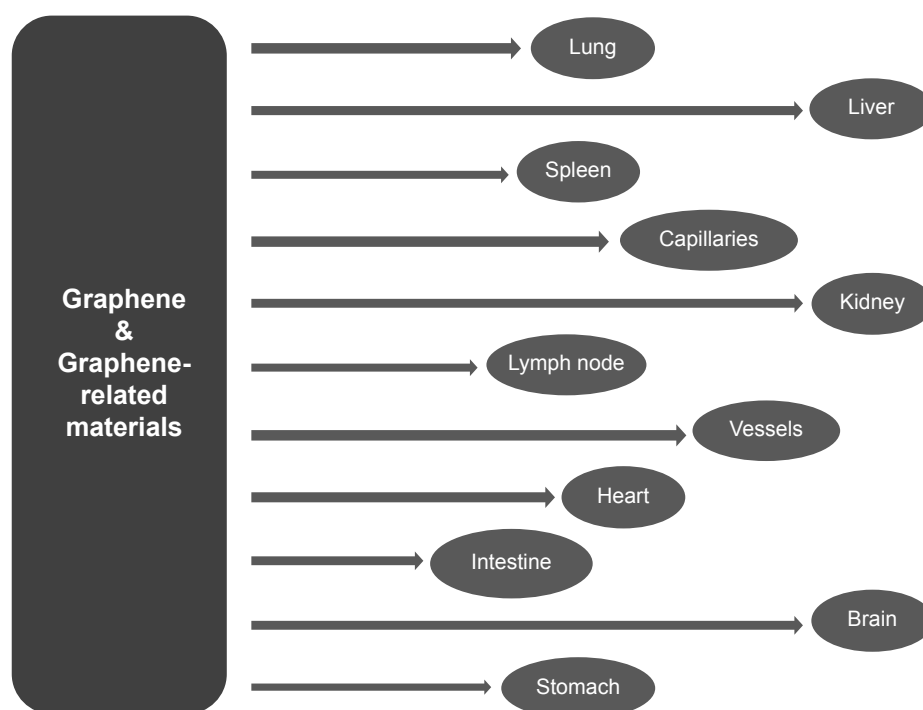


Figure 4 Graphene and graphene-related materials can be used as probes for whole-body functional in vivo bio-imaging of live animals.

the microenvironment via material mechanics,²⁵⁴ substrate topography,²⁵⁵ soluble growth factors,²⁵⁶ and osteogenic inducers, such as dexamethasone and beta-glycerolphosphate.¹⁹¹ Graphene-reinforced chitosan films showed enhanced mechanical and biocompatibility properties in murine fibrosarcoma L929 cell culture.¹⁸⁹ GO-reinforced chitosan scaffolds significantly improved cellular adhesion, proliferation, differentiation, and calcium and phosphate deposition of MC3T3-E1 cells, a mouse preosteoblast cell line.¹⁹⁷ Lee et al¹⁹¹ explored the possibility of using graphene and GO as substrates for MSC adhesion, proliferation, and differentiation, and found that graphene and GO accelerated stem cell growth and differentiation through molecular interactions. Combination of GO and polyvinyl alcohol-based hydrogels significantly enhanced the tensile strength and compressive strength of a composite hydrogel without affecting its cytocompatibility.¹⁷⁸ Nayak et al¹⁹⁹ observed controlled and accelerated osteogenic differentiation of MSCs in the presence of BMP-2. The differentiation of human neural stem cells plays an important role in brain repair and neural regeneration.²⁵⁷ For example, graphene substrate significantly enhanced cell adhesion, neurite outgrowth, and differentiation of human neural stem cells more toward neurons than glial cells.²⁵⁸ Li et al²⁰³ compared the efficiency of tissue culture polystyrene substrates with and without graphene, and they found that graphene films with

excellent biocompatibility significantly promoted neurite sprouting and outgrowth of mouse hippocampal neurons, especially during the early developmental phase. Lu et al²⁵⁹ examined chitosan–polyvinyl alcohol nanofibrous scaffolds with and without graphene for wound healing. They found that graphene-containing nanocomposites show fast wound healing, spontaneous differentiation, iPSC proliferation, and endodermal differentiation.¹⁹³ Ku and Park²⁶⁰ studied the behavior of mouse myoblast C2C12 cells, including adhesion, proliferation, and differentiation, on unmodified, GO-modified, and rGO-modified glass substrates. They found that GO was able to stimulate expression of myogenic protein, enhance myotube formation, and induce expression of differentiation-specific genes.²⁴⁴ Two-dimensional-reinforced polypropylene fumarate nanocomposites such as GO nanoplatelets and molybdenum disulfide nanoplatelets showed better performance as reinforcing agents than one-dimensional nanostructures or single- or multi-walled carbon nanotubes. Calcium adsorption, ALP activity, and growth rate of MC3T3-E1 cells were increased using both GO- and rGO-coated scaffolds compared to non-coated scaffolds.²⁶¹ An artificial matrix (Fn-Tigra), consisting of GO and fibronectin on a pure titanium substrate, enhanced the biocompatibility, cellular behavior, and osteogenic potential of preosteoblasts compared to Ti and Ti-GO (Tigra) substrates. In addition, cell proliferation, viability, and focal

adhesion molecule (vinculin) expression were significantly higher on Fn-Tigra and Tigra than that of cells grown on Ti.²⁶² Lee et al²⁶³ observed that bone marrow-derived MSCs cultured in a solution containing graphene flakes showed increased chondrogenic differentiation. GO sheets composed of GO nanoplatelets and electrospun fibrous meshes of GO–poly-caprolactone composite exhibited significant myoblast differentiation and myotube formation.²⁶⁴ Fabrication of GO-doped poly(lactic-co-glycolic acid) nanofiber scaffolds accelerated the adhesion and proliferation of human MSCs versus pure poly(lactic-co-glycolic acid) nanofibers and induced osteogenic differentiation.²⁶⁵ Primary mouse embryonic fibroblast cells showed significant attachment and survival with amine-reduced GO (Figure 5).

Conclusion and future perspectives

The numbers of studies of graphene and graphene derivatives have increased tremendously in the last 5 years. A large number of studies have been dedicated to developing chemical routes for synthesis and applications of graphene in biotechnology, biomedical engineering, nanomedicine, cancer therapy, tissue engineering, drug delivery, bio-imaging, and biomolecular sensing. However, the number of studies focused on synthesis of graphene using biological molecules is limited. The functional properties of graphene and graphene derivatives depend on size, surface charge, layer number, lateral dimensions, and surface chemistry, and all of these parameters can affect biological systems. The interaction between graphene and biological systems makes graphene an attractive molecule both in academia and industry. In this review, we summarize environmentally friendly approaches for synthesis of graphene using various biological systems, including bacteria, plant extracts, and

small molecules. A number of studies have been published on the potential toxicity of graphene, but many discrepancies between the results remain. These varying results are due to the many factors involved, and the intrinsic physicochemical properties (such as surface functional groups, charges, coatings, sizes, and structural defects) of graphene, as well as differences in size dimensions, functionalization, and purification can all affect its *in vitro* and *in vivo* behavior, as well as its toxicity to biological systems. The properties of graphene are also dependent on the raw materials used for production. Therefore, understanding the toxicity of graphene in biological systems both *in vitro* and *in vivo* is of utmost importance for further development of graphene-based nanomedicine, as well as for providing safety guidelines for all researchers working with this new type of nanomaterial. Another important issue for biomedical applications of graphene is its short- and long-term toxicity. To date, no systematic studies of this subject have been published, and the detailed mechanisms of the cellular toxicity of graphene, *in vitro* and *in vivo*, remain obscure. Another important concern in using graphene in biomedical applications is its biocompatibility. Based on the available literature, we have summarized the possible important aspects of synthesis, toxicity, biocompatibility, and biomedical applications with special reference to cancer therapy, drug delivery, bio-imaging, and tissue engineering. Further, more studies of systematic toxicity versus biocompatibility, particularly in animal models, are required to understand the biological effects and the safety of graphene, before graphene-based nanotherapy can be applied for human welfare. It is necessary to carefully address its solubility, biodegradability, and retention in aqueous solutions. Future studies should focus on functionalization of graphene, excretion of graphene in

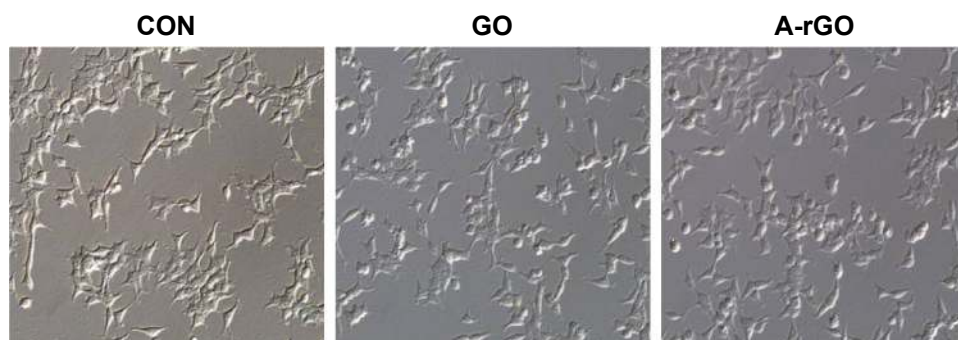


Figure 5 Effect of GO and A-rGO on the survival of MEFs.

Notes: Micrographs showing PMEFC attachment and growth on a non-coated dish (control), a dish coated with GO, and a dish coated with A-rGO. All coated dishes and a control uncoated dish were placed in the same culture conditions and allowed to incubate for 24 hours at 37°C. GO and A-rGO were good substrates for cell growth.

Abbreviations: CON, control; GO, graphene oxide; rGO, reduced graphene oxide; A-rGO, protein-reduced GO; MEFs, primary mouse embryonic fibroblast cells.

animals, and the pharmacokinetics and behavior of graphene in living systems using in vivo animal models. However, graphene and graphene derivatives have made great advancements in the fields of drug delivery and nanomedicine, which may open up new avenues for exciting opportunities to improve human welfare.

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Disclosure

The authors report no conflicts of interest in this work.

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