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Nanotoxicity of Graphene and Graphene Oxide

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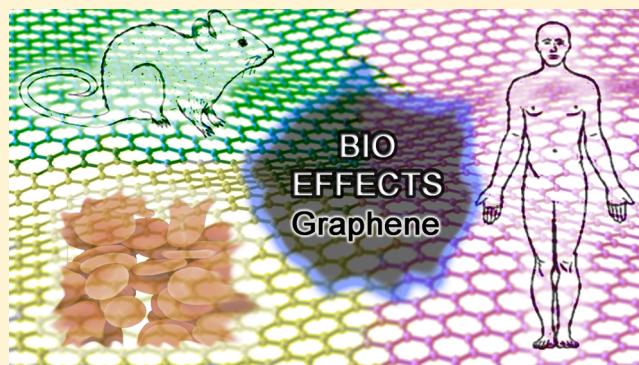
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ABSTRACT: Graphene and its derivatives are promising candidates for important biomedical applications because of their versatility. The prospective use of graphene-based materials in a biological context requires a detailed comprehension of the toxicity of these materials. Moreover, due to the expanding applications of nanotechnology, human and environmental exposures to graphene-based nanomaterials are likely to increase in the future. Because of the potential risk factors associated with the manufacture and use of graphene-related materials, the number of nanotoxicological studies of these compounds has been increasing rapidly in the past decade. These studies have researched the effects of the nanostructural/biological interactions on different organizational levels of the living system, from biomolecules to animals. This review discusses recent results based on *in vitro* and *in vivo* cytotoxicity and genotoxicity studies of graphene-related materials and critically examines the methodologies employed to evaluate their toxicities. The environmental impact from the manipulation and application of graphene materials is also reported and discussed. Finally, this review presents mechanistic aspects of graphene toxicity in biological systems. More detailed studies aiming to investigate the toxicity of graphene-based materials and to properly associate the biological phenomenon with their chemical, structural, and morphological variations that result from several synthetic and processing possibilities are needed. Knowledge about graphene-based materials could ensure the safe application of this versatile material. Consequently, the focus of this review is to provide a source of inspiration for new nanotoxicological approaches for graphene-based materials.



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1. INTRODUCTION

Graphene is composed of sp^2 -hybridized carbon atoms hexagonally arranged in a two-dimensional structure, resulting in a large surface area on both sides of the planar axis.¹ Materials of the graphene family include few-layer graphene, reduced graphene oxide, graphene nanosheets, and graphene oxide (GO).² Compared with carbon nanotubes, graphene-based materials can provide a larger surface area and better dispersibility in most solvents.³ Because of the formation of hydrogen bonds between polar functional groups on the GO surface and water molecules, a stable GO colloidal suspension can be attained,⁴ suggesting advantages for potential biomedical

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applications of GO compared with other carbon-based materials.⁵

Graphenes have been used in diverse nanobiotechnological applications, such as in the environment,⁶ biomedicine,^{7–12} and biotechnology.^{13–16} Consequently, the number of scientific papers based on graphene has increased rapidly since its production in 2004,¹ exceeding 8,500 papers in 2012, as verified by a topic search through the ISI Web of Science. Compared with other carbon-materials, graphene-based systems are younger in development but possess great potential for several biomedical applications.¹¹ However, prior to the use of graphene-based materials, it is imperative to establish a proactive approach for these materials by evaluating their potential toxicity, which is virtually unknown compared with that of other carbon nanostructures, such as carbon nanotubes.² Although the application of graphenes may provide consistent improvements or possible revolutions in the biomedical area, their use is not without risk to human health; therefore, a deeper level of nanotoxicological and human safety studies is required. The level of toxicity that graphene might reach in a biological system and the degree of safety for its use are important to explore.^{2,17} Moreover, multiple graphene forms must be considered because the different types (few-layer graphenes (FLGSs), graphene oxide (GO), reduced graphene oxide (rGO), and graphene nanosheets) possess unique physicochemical properties and, therefore, exert different toxicological effects.¹⁸

Recently, certain studies devoted to the evaluation of the *in vitro* and *in vivo* toxicity of graphenes found contradictory results because toxic and nontoxic effects were simultaneously observed.¹⁹ Therefore, generalized conclusions must be avoided because safety risks associated with graphenes depend on the specific type of material that is analyzed. Generalizations related to graphene toxicities would be inaccurate and possibly misleading.^{3,20,21} The goal of this review is to present and discuss the recent knowledge regarding the toxicity profile of graphenes, especially GO materials.

2. CELL CULTURE STUDIES

Although nanosafety and nanotoxicity have been extensively explored for carbon nanotubes, remarkably fewer studies are available for graphenes. However, there are important studies that investigated the *in vitro* toxicity of graphene-related materials in different cell lines.²² Toxicological aspects related to cytotoxicity and apoptosis induced by graphene in normal human lung cells (BEAS-2B) showed a significant concentration- and time-dependent decrease in cell viability (10–100 $\mu\text{g}/\text{mL}$), as evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) after 24 and 48 h of exposure. An increase in early and late apoptotic cells was also observed compared with control cells.²³ Exposure of human neuronal cells (PC12 cells) to reduced graphene oxide (rGO) (diameter between 100–110 nm and thickness between 3–5 nm) caused an increase in the activation of caspase-3, release of lactate dehydrogenase, and generation of reactive oxygen species (ROS).²⁴ Similarly, graphene has been shown to induce cytotoxic effects and mitochondrial injury in PC12 cells in a dose- and shape-dependent manner.²⁵ Exposure of blood platelets to rGO caused a strong cumulative response and extensive pulmonary thromboembolism.²⁶ rGO interacted with alveolar macrophages and epithelial cells and also generated ROS, leading to inflammation, apoptosis, and an increased rate of mitochondrial respiration.²⁷ However, rGO acting on human hepatoma cells (HepG2 cells) exerted only a moderate effect on protein levels.^{28,29} Zhang and collaborators²⁴ compared the

cytotoxicity level of graphene to that of carbon nanotubes in neuronal PC12 cells. This group found that toxicity was shape- and composition-dependent, with graphene exhibiting an overall lower toxicity than CNTs. Interestingly, the toxicity of graphene was found to be inversely proportional to the concentration,³⁰ exhibiting a higher toxicity compared with CNTs at low concentrations.

Functionalization of the graphene surface with biocompatible polymer chains, drugs, or targeting molecules reduces cellular toxicity of the material compared with its bare counterpart.³¹ Exposure of a human monocytic leukemic cell line (THP-1 cells) to rGO with bovine serum albumin (BSA) resulted in the production and release of inflammatory cytokine (IL1B).³² The cytotoxicity of rGO is significantly reduced by the presence of functionalizing molecules on its surface. Interesting results showed that incubation of several cell cultures, such as lymphoblastoid cells (RAJI),³³ colon cancer cell lines (HCT-116),³⁴ a human ovarian carcinoma cell line (OVCAR-3),³⁵ a glioblastoma cell line (U87MG), and breast cancer cells (MCF-7), with GO capped with polyethylene glycol (PEG), showed no cytotoxicity up to 100 $\mu\text{g}/\text{mL}$.³⁶ PEGylation is possibly the most widely adopted technique used to improve the biocompatibility and solubility of nanomaterials employed in biomedicine. For example, PEGylated nanographene oxide (NGO-PEG), synthesized by oxidizing graphite through a modified Hummer's method, of size ~ 5 to 50 nm, was successfully used for the delivery of the water insoluble cancer drug SN38, a camptothecin (CPT) analogue, leading to a soluble NGO-PEG-SN38 complex.³⁴

In addition to the dependence of toxicity on surface functionalization, the size and dose of graphene also influence cellular toxicity. For example, exposure of A549 cells to GO did not show cell uptake, although size-dependent cytotoxicity and dose-dependent oxidative stress were observed.³⁷ Furthermore, Akhavan and collaborators³⁸ demonstrated that GO sheets and nanoplatelets exerted a size- and concentration-dependent cytotoxicity and genotoxicity toward human mesenchymal stem cells (hMSCs). In this work, rGO and nanoplatelets were prepared by the sonication of covalently PEGylated GO sheets and chemical reduction. The minimum average thickness of the sheets was ca. 0.7 nm, and the authors reported a higher cytotoxicity for rGO nanoplatelets with lower lateral dimensions (11 ± 4 nm), in comparison with larger lateral dimensions (3.8 ± 0.4 μm).³⁸ Exposure of Saos-2 osteoblasts, MC3T3-E1 preosteoblasts, and RAW-264.7 macrophages to GO, which were synthesized from graphite in acidic medium by a modified Hummers method, at a dose of 75 $\mu\text{g}/\text{mL}$ led to cell cycle alterations, apoptosis, and oxidative stress.³⁹ In this work, GO was shown to have an average thickness of 1.8 nm and a hydrodynamic size distribution in the range between 10–120 nm, with a maximum located around 40 nm.³⁹

The ability of macrophages to internalize and remove the graphene materials from the site of deposition serves to enhance their cellular biocompatibility. For example, two phagocytic cell lines were able to internalize nano- and micronized GO with different lateral sizes, showing a selective internalization.⁴⁰ These cells showed no toxicity with an uptake of up to 20 $\mu\text{g}/\text{mL}$ GO. This study revealed that the presence of manganese impurities on GO increases cell toxicity, indicating the importance of purification of the material. Moreover, regarding the uptake of phagocytic cell lines, two different sizes of GO were internalized via different initial cell interactions.⁴⁰ The effect of manganese impurities on GO toxicity is an important aspect that must be considered for all nanostructures to accurately describe the

quality of these materials because the impurities could cause inconsistent conclusions from the nanotoxicity studies. This phenomena was previously described in the case of carbon nanotubes in which the manganese impurities exerted important biological effects.^{41–43}

The intracellular localization of GO was dictated by its size and led to different compartmentalization. Mu and collaborators⁴⁴ in a study with a protein-coated commercial GO (~500 nm) on mouse mesenchymal progenitor C2C12 cells showed the GO internalization by clathrin-mediated endocytosis, whereas larger GO sheets (~1 μm) exhibited uptake by phagocytosis (Figure 1). The GO of both sizes entered lysosomes for

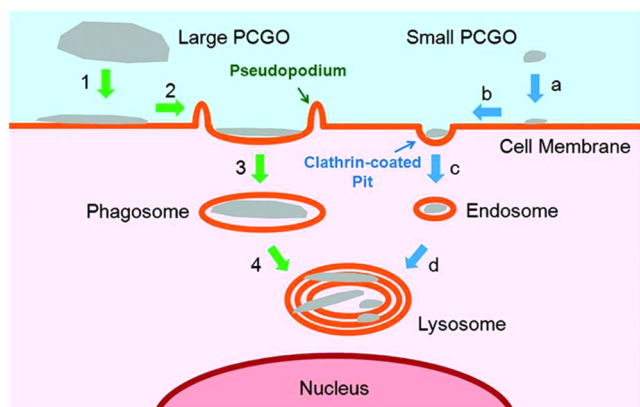


Figure 1. Schematic representation of the cellular uptake of small and large protein-coated graphene oxide (PCGO) nanosheets. Numbers (1–4) and letters (a–d) indicate the different internalization steps of large and small nanosheets into cells, respectively. Reprinted from ref 44. Copyright 2012 American Chemical Society.

excretion. Moreover, using both sizes of GO, almost no inhibition of cell proliferation was found at doses up to 100 $\mu\text{g}/\text{mL}$.⁴⁴ The size range of GO was reported to be from 1.8 ± 0.9 nm to 9.1 ± 7.1 nm, the latter is due to protein coating.⁴⁴

In a study on the possible toxicity of graphene toward macrophage cells, Li and collaborators⁴⁵ demonstrated that pristine graphene induces cytotoxicity on murine macrophage-like cells (RAW 264.7 cells) by the depletion of the mitochondrial membrane potential, thus increasing the generation of intracellular ROS, and by triggering apoptosis through the activation of the mitochondrial pathway. Similarly, GO acts on alveolar macrophages and alveolar epithelial cells by the generation of ROS, yielding inflammation, and resulting in apoptosis of mitochondrial respiration.²⁷ Moreover, GO acting on human fibroblast cells exerts toxicity at doses greater than 50 $\mu\text{g}/\text{mL}$, followed by a decrease in cell adhesion and promotion of cell apoptosis.⁴⁶ In another study, the interaction of commercial pristine graphene dispersed in 1% Pluronic F108 (up to 20 mg/mL) with murine macrophage-like RAW 264.7 cells was investigated.⁴⁷ The thickness of graphene dispersed in 1% Pluronic was found to be 2–3 nm and at a size between 500 to 1000 nm. The results demonstrated that the cells undergo apoptosis in a dose-dependent manner through a mechanism involving a decrease in the mitochondrial potential and an increase in the ROS level.⁴⁷ Murine macrophage-like RAW264.7 cells incubated with GO (~100 $\mu\text{g}/\text{mL}$) were also able to elicit autophagy and the expression of Toll-like receptors associated with inflammatory responses.⁴⁸ Dendritic cells treated with GO (up to 25 $\mu\text{g}/\text{mL}$) were affected in their functional activity (antigens inhibition),⁴⁹ and this effect was associated with a down-regulation of

intracellular levels of one unit of the immune proteasome responsible for antigen processing in these cells.

The cytotoxicity of GO and biogenic rGO was evaluated on human skin fibroblasts (CRL-2522). Unfortunately, the MTT assay was found to be inappropriate for this analysis because graphene reacts with the MTT reagent, displaying a false-positive result.⁵⁰ To avoid this effect, a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay was used instead of the MTT assay. The results showed that both GO (synthesized from graphite powder using a modified version of Hummers and Offeman's method) and biogenic rGO (synthesized by *Bacillus marisflavi* biomass) exhibited toxicity on MCF-7 cells in a dose-dependent manner (up to a dose >60 $\mu\text{g}/\text{mL}$), thus decreasing the cell viability, increasing the ROS generation, and releasing lactate dehydrogenase.⁵¹ Atomic force microscopy revealed that GO exhibit flat sheets with an average thickness of about 0.43 nm, indicating the formation of single-layered GO nanosheets. However, biogenic rGO was found to be thicker (~4.23 nm), indicating the presence of biomass on the GO surface.⁵¹ In another study, Ruiz and collaborators⁵² observed the attachment and proliferation of mammalian colorectal adenocarcinoma cells (HT-29 cells) upon exposure to GO films, indicating no significant toxicity. Moreover, GO films at a concentration of 20 $\mu\text{g}/\text{mL}$ decreased the cell viability by 20%, and at a concentration of 50 $\mu\text{g}/\text{mL}$, the cell viability decreased to 50%.⁵² Akhavan and collaborators⁵³ compared the efficiency of glucose-functionalized graphene, reduced-graphene, and hydrazine-reduced GO for photothermal therapy in cancer cells. The authors observed that the reduced-GO suspension and glucose-functionalized GO exhibited biocompatible properties and higher photothermal therapy efficiencies compared with those of hydrazine-reduced GO. Furthermore, Cheng and collaborators⁵⁴ demonstrated a dose-dependent cytotoxicity in human umbilical vein endothelial cells (HUVEC) toward GO. Their group observed no toxicity upon cell exposure to doses greater than 100 $\mu\text{g}/\text{mL}$ functionalized rGO, although a higher level of cytotoxicity was observed in the case of GO reduced with hydrazine.

Recently, Jaworski and collaborators⁵⁵ studied the effects of graphene platelets (GPs) in human glioma cell lines (U118, U87) and observed that GPs were toxic to both cell lines. GPs at a concentration of 100 $\mu\text{g}/\text{mL}$ led to a high apoptosis rate (99%) in U118 cells and a low necrosis rate (0.2%) compared with 68% and 24%, respectively, in U87 cells. This difference was explained by the chemical nature of the cell surface membranes and the expression of the genes in each cell line.

The hemocompatibility of graphene and GO with human primary blood components is an important toxicological aspect for graphene-based materials. A comparison of the hemocompatibility of graphene and GO showed that graphene exerted a slightly higher cytotoxic effect due to its strong hydrophobic interaction with cell membranes,⁵⁰ although both nanomaterials showed an insignificant hemolytic effect (up to 75 $\mu\text{g}/\text{mL}$) and insignificant levels of coagulation.⁵⁶ However, another report showed that GO at 2 $\mu\text{g}/\text{mL}$ exerted thrombotic potential.^{26,57} This apparent contradiction is possibly a result of the use of GO with different morphologies and chemical structures.

The investigation of genotoxicity of nanomaterials is important because there is a close correlation between DNA damage and mutation and cancer.⁵⁸ Fewer studies have investigated the genotoxicity (DNA damage) of nanomaterials compared with the number of cell death studies. Recently, Qiao and collaborators⁵⁹ compared the genotoxicity of different

nanomaterials, such as iron oxide (Fe_3O_4), titanium dioxide (TiO_2), silicon dioxide (SiO_2), zinc oxide (ZnO), indium (In), tin (Sn), core-shell zinc sulfate-coated cadmium selenide (CdSe@ZnS), GO, and carbon nanotubes, toward human fibroblast cells. All studied nanoparticles were less than 50 nm in each dimension, and GO showed 2 μm in lateral size and 1.5 nm in thickness. The authors observed that the different nanomaterials caused considerable variation in DNA damage. Each tested nanomaterial showed a concentration-dependent genotoxic effect, although graphene was found to cause the most DNA damage. The lowest tested graphene concentration (1 $\mu\text{g}/\text{mL}$) caused genotoxicity, whereas nanoparticles of SiO_2 , ZnO , TiO_2 , Sn, and carbon nanotubes induced DNA damage only at higher concentrations (100 $\mu\text{g}/\text{mL}$).⁵⁹ Therefore, more studies on the genotoxicity of graphene-related materials are necessary.

3. IN VIVO STUDIES

The number of *in vivo* studies based on tissue distribution and excretion of graphene is gradually increasing. GO administration in mice induces chronic toxicity and lung granuloma death.⁴⁶ In addition, dose-dependent pulmonary toxicity, granulomatous lesions, pulmonary edema fibrosis, and inflammatory cell infiltration were also found after GO administration.^{27,60} A pulmonary inflammatory response was also observed after BSA-capped graphene administered in rats.³²

Zebrafish has been established as an important preclinical model for *in vivo* toxicity studies of nanomaterials due to its close homology with the human genome.⁶¹ Furthermore, zebrafish embryos are more sensitive to chemical agents compared with adult organisms.⁶¹ Gollavelli and Ling⁶² studied the *in vivo* toxicity of graphene to *Danio rerio* (zebrafish) embryos microinjected with multifunctionalized graphene (coated with polylactic acid and fluorescein *o*-methacrylate). The authors did not observe significant abnormalities or changes in the survival rate of fish embryos, although graphenes were found to be largely biodistributed in zebrafish. In a similar study, the biotoxicity of self-assembled nanosized complexes composed of naphthalene-terminated PEG and anticancer drugs (curcumin or doxorubicin (DOX)) was investigated using the embryo zebrafish model.⁶³ The results showed that the graphene complexes were not toxic toward the development from the embryo to the larvae stages. This *in vivo* biocompatibility can be explained by the presence of capping functionalities on the graphene surface. Because graphene is not a degradable material, its solubility and biocompatibility can be improved by chemically coating it with hydrophilic polymers, such as PEG, which will decrease its hazard.⁶³

Studies based on the uptake of PEG-coated graphene nanosheets in mice and subsequent photothermal treatment of cancerous tumors did not show any adverse toxic effects.^{30,64} PEGylation of GO reduces the toxic effects in mice, and similarly, no severe toxicity was measured *in vivo* upon administration of GO as a component in injectable hydrogels for tissue engineering.⁶⁵ A recent study characterized the fate of GO and PEGylated GO after oral feeding and intraperitoneal (i.p.) injection into healthy mice.⁶⁶ PEGylated GO materials showed no uptake via oral administration, indicating limited intestinal absorption of the material, with almost complete excretion. In contrast, upon i.p. injection in mice, PEGylated GO was found to accumulate in the liver and spleen. The authors assumed that PEGylated GO materials were engulfed in a size- and surface-capping dependent process by phagocytes after i.p. injection.⁶⁶

Zanni and collaborators⁶⁷ investigated the impact of graphite nanoplatelets on the living organism model *Caenorhabditis elegans* (nematode). The authors did not report toxicity (measured by longevity and reproductive capacity end points) for graphite nanoplatelets, although a homogeneous distribution of the nanomaterial was found inside the nematode organism (Figure 2). As shown in Figure 2, graphite nanoplatelets in

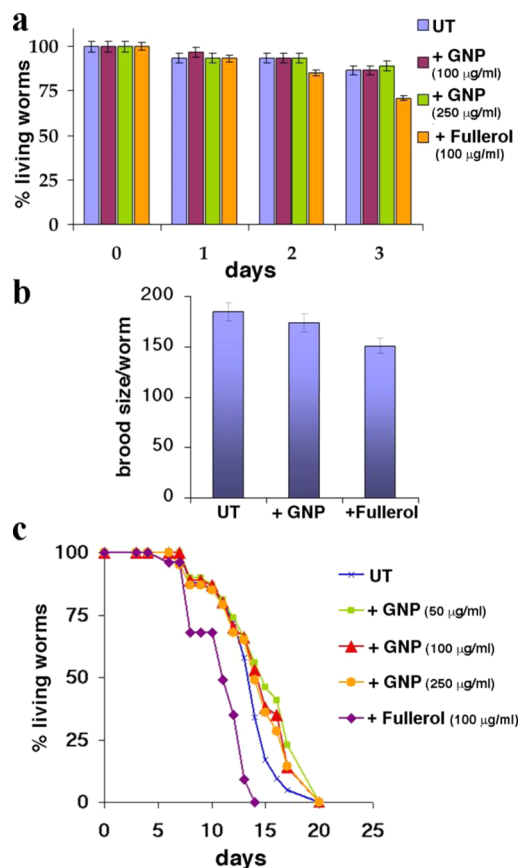


Figure 2. (a) *In vivo* toxicity of graphite nanoplatelet (GNP) suspensions on *C. elegans* worms. The worms were incubated with different concentrations of GNPs for 3 h, and the nematode survival was monitored for 3 days. A suspension of fullerol nanoparticles (100 $\mu\text{g}/\text{mL}$) was used as a positive toxicity control. (b) Average brood sizes per nematode worm exposed or not to suspensions of GNPs (250 $\mu\text{g}/\text{mL}$) or fullerol (100 $\mu\text{g}/\text{mL}$). (c) Chronic toxicity assay for GNPs or fullerol suspensions on *C. elegans* worms. Newly hatched *C. elegans* were seeded onto NGM plates supplemented with *E. coli* and were treated or not with GNP suspensions at the indicated concentrations. Reprinted from ref 67. Copyright 2012 American Chemical Society.

different concentrations did not increase nematode mortality, indicating the absence of acute toxicity *in vivo*. However, another study showed that a graphene nanosheet with sharp edges⁶⁸ caused considerable damage to the cell membrane of bacteria, providing a useful antibacterial property. Moreover, hydrophilic carboxyl-functionalized graphenes have the ability to be internalized in cells without any toxic effects, which is in contrast to hydrophobic pristine graphene.⁶⁹ The toxicity of GO was observed to be dose-dependent in both human and animal cells, displaying little to no effect for low and medium doses in mice.⁴⁶ Significant pathological changes, including inflammatory cell infiltration, pulmonary edema, and formation of granulomas, were found using doses of 10 μg of GO per gram of body weight. However, GO showed good biocompatibility

with red blood cells at very low doses, whereas hemolysis was induced at 80 $\mu\text{g/mL}$ GO.⁷⁰

The intravenous administration of GO or amine-derived GO (GO-NH₂) into a mouse (250 $\mu\text{g/kg}$ body weight) led to extensive pulmonary thromboembolism, which is consistent with the highly potent thrombogenic nature of GO, as previously shown *in vitro*; however, amine-GO (GO-NH₂) was found to be less toxic compared with its unfunctionalized counterpart. Moreover, administration of rGO and GO into mice showed that rGO is less effective at activating platelets compared with GO, which may be correlated with the reduced charge density on the graphene surface. Therefore, these nanomaterials should be critically evaluated due to their thrombogenic potential.^{25,57} In an intraocular biocompatibility and cytotoxicity study of GO, both *in vitro* and *in vivo* results suggested that GO has good intraocular biocompatibility, with minimum effects on cell morphology, cell viability, membrane integrity, and apoptosis.⁷¹

Analysis of the combined data provides important information regarding the toxicity of graphene-related materials. Because several results are contradictory, it is important to avoid generalizations about the data because of the significant variability of the material under study. Using these data, it is essential to compare the different types of graphenes (functionalized or not) and to correlate their biological impact to their physicochemical characteristics or structural modifications and, thus, avoid generalized conclusions about the toxicity of graphenes. In contrast, many graphenes are considered not toxic.⁷²

4. ANTIMICROBIAL STUDIES

Recent studies indicate that graphene-based materials could be used in antimicrobial products because of their versatility.⁶⁸ Hu and collaborators⁷³ prepared macroscopic freestanding GO and rGO papers with strong antibacterial effects. Because of the easy scalability and low cost, GO materials might be used in important applications in environmental and clinical fields, as suggested by the authors.

In other studies, GO was tested against Gram-negative *E. coli* and Gram positive *S. aureus*, and the results showed that *E. coli* was found to be resistant to GO.⁶⁸ However, Hu et al.⁷³ and Feng and Liu³⁵ found a useful bactericidal effect of GO on *E. coli* because it caused bacterial membrane damage. Direct interaction of bacteria with graphene sheets induced the loss of bacterial membrane integrity and glutathione oxidation, suggesting that the GO antimicrobial action contributes to both membrane disruption and oxidative stress. The data suggest that the physicochemical characteristics of graphene materials appear to play an important role in their efficiency for killing bacteria, although these results were questioned by Ruiz et al.⁵² Upon incubation of *E. coli* with GO, the bacteria grew faster by forming dense biofilms around the suspended nanomaterial, and only the addition of silver nanoparticles led to antimicrobial activity.⁷⁴ Similar results were observed by Wang et al.⁷⁵ on *Shewanella* growth. When GO and reduced GO were tested against Gram-negative *E. coli* and Gram-positive *S. aureus*, a more efficient antimicrobial activity was observed for rGO compared with GO.⁶⁸ Similar results were found by Liu and collaborators.¹⁷

Liu et al.⁹ systematically investigated the antibacterial effects of different GO suspensions with different lateral sizes (of more than 100 times) and with distinct size distributions. The antibacterial activities of GO sheets toward *E. coli* cells were found to be more potent in larger GO sheets than in smaller sheets.

The authors also observed different time- and concentration-dependent antibacterial activities. Their results suggested that the size-dependent antibacterial activities of GO sheets could not be assigned to the different aggregation states or to the oxidation capacity because GO sheets were well dispersed independent of lateral sizes, and their antioxidant activities were similar. Larger GO sheets could most likely cover cells more easily, which may block their active sites on membranes. In addition, smaller GO sheets inefficiently adhered to the bacterial surfaces, exhibiting a weaker antibacterial activity.⁷⁶

Further studies conducted by Carpio et al.⁷⁷ and Santos et al.⁷⁸ showed that the antibacterial activity of GO was maintained or improved by its dispersion on poly-*N*-vinylcarbazole and through its electrodeposition on a substrate, and it did not exhibit cytotoxic effects on mammalian cells in a dose of 1000 $\mu\text{g/mL}$. Interaction of GO with *E. coli* led to a reduction of the graphene sheets and the presence of rGO. After the bacterial reduction of GO, an inhibition of bacterial proliferation and surface detachment was observed.⁷⁹

Strong antibacterial activity of GO, synthesized by the Hummers method, was reported on *Klebsiella* sp. and *Staphylococcus* sp. bacterial species, in which the inhibition zone was a concentration-dependent parameter.⁸⁰ Moreover, the antibacterial activity of GO nanosheets toward Gram-negative bacteria *Escherichia coli* and Gram-positive bacteria *Streptococcus iniae* was investigated by the colony counting method, and the results showed that GO nanosheets were more effective toward Gram-positive bacteria compared with Gram-negative bacteria. The authors suggested that the antibacterial mechanism involved the generation of reactive oxygen species.⁸¹

In another study, the antibacterial activity of rGO and GO, prepared from nature graphite powders by the Hummers method, suspended in different dispersants was evaluated against *Xanthomonas oryzae pv oryzae*, a representative phytopathogenic bacterium that causes infections in rice.⁸² The average size of the nanosheets was found to be in the range of 300–600 nm, as determined by dynamic light scattering, and atomic force microscopy revealed that GO were flat sheets with an average thickness of 0.76 nm, while rGO presented a sheet thickness of 1.59 nm. Moreover, the results showed a superior bactericidal effect upon bacterial exposure to GO (250 $\mu\text{g/mL}$) compared with rGO and bismethiazol, a common bactericide, with a killing rate of 94.48%, 36.31%, and 13.3%, respectively. The high efficiency of GO for inactivating the bacteria was presumably due to its extremely sharp edges and the generation of reactive oxygen species.⁸² Because of the promising antibacterial action of graphene-related materials, GO was loaded in cotton fabrics through various methods, and the composites showed high antibacterial properties with minimal skin irritation.⁸³

In addition, rGO (up to 500 $\mu\text{g/mL}$) displayed antifungal activity on the nonpathogenic fungus *Aspergillus oryzae* and on the pathogenic fungi *Aspergillus niger* and *Fusarium oxysporum*.⁸⁴ Figure 3 shows the logarithm of the rGO concentration versus the mycelial growth inhibitory activity (%). The IC₅₀ values for rGO were found to be 50, 100, and 100 $\mu\text{g/mL}$ against *F. oxysporum*, *A. niger*, and *A. oryzae*, respectively. The higher antifungal activity against *F. oxysporum* could be explained by considering the ease of attachment of rGO to the external *F. oxysporum* cell wall through the hydroxyl oxygen species of glycoproteins.⁸⁴ The antifungal activity of rGO against non-pathogenic microorganisms, such as *Aspergillus oryzae* (Figure 3), is an important environmental concern.

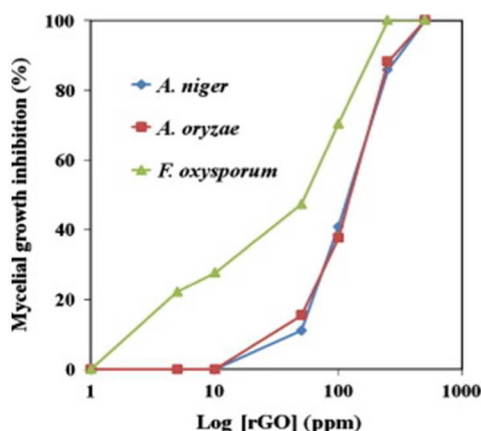


Figure 3. Plots of the logarithm of the rGO concentration ($\mu\text{g/mL}$) versus the mycelial growth inhibitory activity (%). Reprinted with permission from ref 84. Copyright 2012 Elsevier.

5. ENVIRONMENTAL TOXICITY

Recently, the environmental and biological toxicities of graphene and/or graphene composites applied in environmental remediation have been discussed.⁸⁵ Plants, such as cabbage, tomato, red spinach, and lettuce, treated with graphene ($>500 \mu\text{g/mL}$) showed a significant inhibition of growth, biomass levels, and the number and size of leaves, in a dose-dependent manner. Moreover, the effects of graphene on root and shoot growth, biomass, shape, cell death, and ROS formation were also evaluated by Begum et al.⁸⁶ Graphene induced negative effects, such as a concentration-dependent increase in ROS, cell death, and visible symptoms of necrotic lesions on cabbage, tomato, and spinach. On the basis of root morphology studies, the authors suggested that plant cell death induced by graphene treatment might occur either by apoptosis or by necrosis. These facts indicate that the potential toxic effect of graphenes on plants may largely depend on the graphene dose, exposure time, and the plant species. Therefore, this topic deserves further attention.⁸⁶

To assess physiological responses, such as biomass accumulation, few-layer graphene materials were introduced into Murashige and Skoog (MS) media ($50 \mu\text{g/mL}$) along with tomato seeds with a sterilized surface, and the germination and growth were followed.⁸⁷ The authors observed that few-layer graphene carbon structures did not significantly affect plant growth rates, most likely because of their inability to penetrate plant tissues.

Short-term and high-load effects of GO on microbial functions related to biological wastewater treatment processes showed toxic effects on microbial communities in a dose-dependent manner, especially in concentrations greater than 50 mg/L . Many biological activities were significantly impacted by the presence of GO in an activated sludge. The authors suggested that the generation of reactive oxygen species (ROS) could be one of the responsible mechanisms for the toxic effect of GO.⁸⁸ In this sense, graphene is a promising material for environmental applications, and its phytotoxicity should be further investigated.⁶

6. TOXICITY MECHANISMS

Although the mechanisms responsible for graphene and GO toxicity have been discussed previously,⁵¹ no conclusions have been drawn that are sufficient to establish risk assessments or regulations.⁸⁹ Graphene is a unique nanomaterial compared with

spherical nanoparticles and one-dimensional nanotubes due to its two-dimensional structure.

Many reports have proposed that oxidative stress is one of the mechanisms involved in the toxic effects of carbon nanomaterials.^{2,24,37,90} The oxidative stress in target cells is caused by the generation of reactive oxygen species (ROS).² Antioxidant enzymes, such as superoxide dismutase or glutathione peroxidase, are able to reduce and eliminate ROS. If homeostasis is not achieved, cellular macromolecules, such as proteins, DNA, and lipids, can be damaged.²

Cell membrane damage through physical interaction with graphenes possessing sharp edges is another possible mechanism of toxicity.^{68,73} Furthermore, due to its hydrophobic surface, graphene can significantly interact with cell membrane lipids, causing toxicity.²

Bacterial cells interacting with GO and rGO exhibited toxicity, which may be due to the loss of membrane integrity, and the proposed toxicity mechanism includes initial cell deposition on graphene-based materials and membrane stress caused by direct contact with sharp nanosheets.¹⁷ Gurunathan et al.⁹⁰ previously demonstrated that graphene nanomaterials (GO and rGO) interacted with cells, inducing toxicity in a concentration-dependent manner. The efficiency of GO at inactivating bacteria was assigned to its sharp edges and the generation of reactive oxygen species.⁸² Similarly, the generation of reactive oxygen species could be one of the responsible mechanisms for the observed toxic effects of GO on the biota of activated sludge.⁸⁸

Toxicity also depends on the physicochemical properties of graphene-based materials, such as the density of the functional groups, size, conductivity, and chemical nature of the reducing agent used for deoxygenation of GO, as well as on the cell types exposed to the materials.⁹⁰ Robinson et al.³⁶ studied the *in vitro* toxicity of rGO sheets (single-layered nano-rGO sheets $\sim 20 \text{ nm}$ in average lateral dimension) noncovalently functionalized with PEG polymer chains toward human epithelial breast cancer cells. In this study, a low level of toxicity was observed. Similar results were reported by Wojtoniszek et al.,⁹¹ in which L929 mouse fibroblasts cells were exposed to GO and reduced GO functionalized with different dispersants, such as PEG, Pluronic P123, or sodium deoxycholate (DOC). The authors showed that the cell toxicity depends on the type of dispersant and the concentration of the nanomaterials in the suspensions. Similarly, oxidized graphene nanoribbons coated with PEG-derived structures showed increased solubility and stability, and toxicity screening (with alamar blue, neutral red, trypan blue, LDH release, a clonogenic assay and a live cell assay) on four cell lines (HeLa, MCF7, SKBR3, and NIH3T3) indicated that this nanomaterial has a dose- and time-dependent effect and differential cytotoxic effects on the four cell lines.⁹² In this work, oxidized graphene nanoribbons were synthesized via longitudinal unzipping of multiwalled carbon nanotubes and display an average width of $125\text{--}220 \text{ nm}$ and lengths between $500\text{--}2500 \text{ nm}$.⁹² These results show that the toxicity mechanism of graphene-based materials on cells is highly dependent on the surface of the nanomaterial.

7. METHODOLOGIES EMPLOYED TO EVALUATE GRAPHENE AND GRAPHENE OXIDE TOXICITIES

The most common cytotoxicity assays to evaluate the toxicity of graphene-related materials are MTT, caspase-3,7 assays, LDH membrane integrity assay, generation of reactive oxygen species (ROS), hemolysis, cell adhesion and morphology, platelet activation, apoptosis assay, cytokine detection, genotoxicity,

and hemocompatibility.^{5,23,24,32,37,38,50,56} It must be highlighted that the genotoxicity assay is considered an important indicator of toxicity since there is a close relationship between damages to DNA and mutations that may lead to cancer.⁵⁸ Among all assays, MTT is by far the most popular one to characterize the cytotoxicity of materials. However, this assay was reported to be problematic in studies with carbon nanotubes and as a consequence to graphene-related materials.^{5,50} Alternatively, the WST-8 assay is preferred in the place of traditional MTT.⁵⁰ Therefore, caution must be taken to choose the most appropriate cytotoxic assay to evaluate the toxicity of graphene-related materials to avoid false-positive results.

The great majority of *in vivo* studies of graphene-related nanomaterials is based on evaluation of tissue distribution (bioaccumulation) and excretion. Zebrafish is considered the most used animal model to evaluate the *in vivo* toxicity of graphene-related materials.⁶¹ It should be considered that the administration route is an important parameter that impacts the toxicity of nanomaterials, and it may be further explored.⁶⁶ It is clear from the recent literature that due to the increasing importance of graphene-related materials, there is a need for more detailed and accurate *in vitro* and *in vivo* studies of toxicity of the graphene family.

8. CONCLUSIONS AND PERSPECTIVES

In the past few years, graphene-related nanomaterials have emerged as promising scaffolds for a wide range of technological and biomedical applications, including drug delivery, biosensing, tissue engineering, and diagnosis. However, to ensure the safe use of graphene materials in biomedical applications, evaluation of the safety and potential risks of these materials is mandatory. Currently, limited information about the *in vitro* and *in vivo* toxicity of graphene is available, and more studies are required.

Graphene-based materials are unique and possess significantly different properties than spherical nanoparticles and one-dimensional carbon nanotubes. This review reveals that the toxicity of graphene is dependent on the graphene surface (the chemical structure or the nature of the functionalized coatings), size, number of layers, cell type, administration route (for *in vivo* experiments), dose, time of exposure, and synthesis methods. The toxicity profile of graphenes will depend on several parameters, and generalizations should be avoided. In addition, systematic investigations should be carefully performed to correlate each of these parameters to the biological event induced by graphenes.

The current literature proposes that the generation of reactive oxygen species in target cells is the most important cytotoxicity mechanism of graphene. Further studies are required to better understand the toxicity pathways, in particular those that focus on the investigation of cellular interactions of graphene materials with cell membrane lipids on a molecular level.

Overall, an important conclusion that can be postulated is that small and hydrophilic graphene nanomaterials (in particular, those capped with biocompatible molecules) tend to form a stable colloid dispersion, avoiding aggregation and, therefore, are more apt to be internalized and removed/excreted from the application site. Moreover, colloidal dispersions of individualized graphene sheets (or graphene oxide and its derivatives) can be more easily engineered without metallic impurities compared with several types of carbon nanotubes, making graphene-based materials promising candidates for biomedical applications. In addition, graphene nanostructures are not fiber-shaped and

theoretically offer significant advantages in terms of safety over inhomogeneous dispersions of carbon nanotubes.

Toxicological studies should consider the purity (quality) of the sample, especially the presence of oxidative debris formed during the early stages of synthesis and/or during the functionalizing process, which largely alters the surface microchemical environment of graphene and GO.⁹³ In addition, the nonmolecular behavior of graphenes must be considered in nanotoxicological models and protocols because of the intrinsic variation of the graphene characteristics, such as size, morphology, and chemical structure related to the nature of this material,⁹⁴ in addition to oxidative methods used to prepare GO.⁹⁵

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Notes

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ABBREVIATIONS

GO, graphene oxide; FLGSs, few-layer graphenes; rGO, reduced graphene oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt; ROS, reactive oxygen species; HepG2 cells, hepatoma cells; CNTs, carbon nanotubes; PEG, polyethylene glycol; NGO-PEG, PEGylated nanographene oxide; CPT, camptothecin; hMSCs, human mesenchymal stem cells; HUVEC, human umbilical vein endothelial cells; GPs, graphene platelets; DOX, doxorubicin; MS, Murashige and Skoog; DOC, sodium deoxycholate

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