Assessment of the toxic potential of graphene family nanomaterials

Xiaoqing Guo*, Nan Mei
Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA

1. Introduction
Advancements in the field of nanotechnology have the potential for improving diagnostic, therapeutic, and preventive medical products, as well as in applications for food packaging, processing, and preservation. The United States Food and Drug Administration has already approved some nanotechnology-based products and expects a significant increase in the use of nanomaterials in drugs, devices, biologics, cosmetics, and food [1]. However, the rapid development and commercialization of nanoscale products in recent years have increased the possibility of human exposure to engineered nanomaterials through four distinct entry routes: inhalation, ingestion, dermal penetration, and injection or implantation [2,3]. For safe applications of the nanoscale products, it is essential that thorough safety assessments be conducted in order to protect human health and the environment [4].

The opinions expressed in this article are those of the authors and do not necessarily reflect those of the US Food and Drug Administration.

* Corresponding author. Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079, USA.
E-mail address: xiaoqing.guo@fda.hhs.gov (X. Guo).
1021-9498 Copyright © 2014, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. Open access under CC BY-NC-ND license.
http://dx.doi.org/10.1016/j.jfda.2014.01.009
Nobel Prize in Physics in 2010 "for groundbreaking experiments regarding the two-dimensional material graphene".

Graphene, one of the carbon nanomaterial allotropes, is a single-atom-thick, two-dimensional sheet having sp²-hybridized carbon atoms arranged hexagonally. It is the thinnest possible configuration of carbon molecules, and is a basic building block for other graphitic materials such as graphite, large fullerenes, and carbon nanotubes (CNTs) [6]. Graphene has unique physicochemical properties including a high surface area, extraordinary electrical and thermal conductivity, and strong mechanical strength [6,7]. The excellent electronic transport properties and high surface-to-volume ratios endow it with unique mechanical and rheological properties, and resistance to degradation [8]. The two active parts, surfaces and edges, facilitate graphene attaching to biological molecules and adhering to cells [9].

Since 2004, graphene has become a “superstar” in the field of nanotechnology. Graphene is a promising platform for numerous applications including in nanoelectronics and energy technology (supercapacitors, batteries, and composites) as sensors, and for biomedical applications (drug/gene delivery, biosensors, cell and tumor imaging, adsorption of enzymes, and cancer photothermal therapy) [10–15]. These widespread applications have attracted interest in the manufacturing of graphene and its derivatives, referred to as graphene-family nanomaterials (GFNs). GFNs include single- or few-layer graphene, graphene nanosheets, graphene ribbons, graphene oxide (GO), and reduced graphene oxide (rGO) [16]. Subsequent development of covalently and non-covalently functionalized GFNs improved their biocompatibility, stability, and reduced their toxic side effects in the physiological environment [17–19].

Reports indicate that the market projections for graphene-based products will reach $675 million by 2020 [20]. This review describes the most recent reports on the toxicological activity of GFNs both in vitro and in vivo. Also discussed are the effects of GFN functionalization on diminishing their toxic interaction with cells, and the potential mechanisms for GFNs-induced toxicity. The material properties relevant to the biological effects and the applications of GFNs in the field of drug delivery and food preparation are also covered in this article.

2. The properties and bioapplications of GFNs

GFNs vary in shape, size, surface area, layer number, lateral dimensions, surface chemistry, stiffness, defect density or quality of the individual graphene sheets, and purity; and all these properties significantly influence the interaction of GFNs with biological systems [16]. Generally, GFNs with small size, sharp edges, and rough surfaces easily internalize into the cell as compared to larger, smooth GFNs. GFNs, particularly monolayer graphene, have the theoretical maximum surface area because every atom lies on the surface, providing an extremely high capacity for drug delivery. The specific surface area and bending stiffness depend on the number of layers. For biological molecules, the more layers of GFNs, the lower the adsorptive capacity. The lateral dimensions of GFNs, with a range of 10 nm to >100 μm, affect cell uptake mode-of-action, renal clearance, blood–brain barrier transport, and many other biological interactions [21,22]. The surface chemistry varies greatly among the members of GFNs even before any surface modification; it determines their hydrophilicity or hydrophobicity, stability, and dispersibility in physiological conditions [15]. Furthermore, because graphene can be synthesized by various methods, e.g., mechanical or chemical exfoliation of intercalated bulk graphite [6,23], it is inevitable that GFNs contain some impurities such as chemical additives or residual intercalants, including nitrate, sulfate, and peroxide [16]. Compared to as-made GO, highly purified GO produces negligible negative effects in vitro and in vivo [24], indicating a need to consider the impurities for the biological effects studies.

3. Applications in drug delivery and food

Due to graphene’s unique properties, there has been increasing interest in using graphene and its derivatives for drug delivery [19]. The planar structure and ultra-high surface area (2600 m²/g) of graphene facilitate molecular loading and bioconjugation [25]. GFNs, specifically GO and graphene, have been evaluated as novel nanocarriers for a variety of therapeutic applications, including the delivery of conventional drugs, because their use may alleviate problems due to multidrug resistance and nonspecific targeting [26,27]. Previous in vitro and in vivo studies have confirmed that GO is highly efficient in the targeted delivery of the anticancer drugs, doxorubicin and SN38 (a camptothecin analogue), and it is a promising platform for cancer therapy involving insoluble drugs [28,29]. Subsequently, development of various functionalized GFNs has improved the biocompatibility, solubility, and drug delivery efficiency [30–34]. For example, transferrin-conjugated polyethylene glycol (PEG)-GO displayed greater intracellular delivery efficiency and stronger cytotoxicity against C6 glioma cells [33], and a doxorubicin-loaded targeting peptide-modified mesoporous silica-coated graphene nanosheet provided synergistic chemo-photothermal targeted therapy for gliomas [34].

Recently, development of a dual-targeted (magnetic and biological) drug delivery system has improved efficiency. Triple functionalized GO-doxorubicin, encapsulated by Fe₃O₄ and folic acid-conjugated chitosan, exhibited high loading efficiency and targeted drug delivery to the tumor area [35]. In addition, delivery of more than one anticancer drug by GO also has been reported [14]. Controlled loading of both doxorubicin and camptothecin onto folic acid-conjugated GO via π–π stacking and hydrophobic interactions resulted in both target specificity and much higher cytotoxicity to MCF-7 cells than conjugated GO loaded with either drug alone [14]. The most recent in vivo studies also demonstrate an enhanced anticancer effect of functionalized GFNs as a drug delivery system. Transferrin conjugated PEG-GO-doxorubicin delayed tumor volume expansion and increased the survival of a C6 glioma-bearing rat model [33]. Intravenous injection of 40 mg/kg doxorubicin loaded on cholesteryl hyaluronic acid modified rGO in tumor-bearing mice exhibited a higher loading capacity, increased colloidal stability under physiological conditions.
conditions, improved safety, and increased accumulation in tumors when compared to rGO/doxorubicin [36]. A zebrafish model demonstrated that a GO/NP–curcumin complex was exerted quickly from the zebrafish body and had nearly no influence on the development of zebrafish from embryos (the stage of 12 cells) to larvae (age, 4 days), suggesting the safe bioapplication of graphene-based therapeutic modalities [37].

Applications of GFNs related to food include their use for food packaging, water purification, and sensors for detecting contamination. Graphene nanoplates can form heat resistant food packaging, water purification, and sensors for detecting bioapplication of graphene-based therapeutic modalities [37].

Stage of 12 cells) to larvae (age, 4 days), suggesting the safe influence on the development of zebrafish from embryos (the excreted quickly from the zebrafish body and had nearly no influence on the development of zebrafish from embryos (the stage of 12 cells) to larvae (age, 4 days), suggesting the safe bioapplication of graphene-based therapeutic modalities [37].

4. Toxicity of GFNs

Reports indicate that GFNs exert measurable cytotoxicity in both in vitro and in vivo studies in various types of bacteria, mammalian cells, and animal models. Most published studies have evaluated GO and rGO due to their better solubility/dis-

speribility/stability in water and under physiological condi-
tions compared to other GFNs.

4.1. Toxicity in bacteria

Recent studies have investigated GFNs toxicity in both bac-
teria and fungi. Graphene effectively inhibited the growth of Gram-negative Escherichia coli and Gram-positive Bacillus subtilis at a concentration of 1 mg/mL [42]. Two water dispersible graphene derivatives, GO and rGO nanosheets, inhibited the growth of E. coli with minimal cytotoxicity [43]. In the colony-forming assay, more than 90% of the bacteria lost viability following a 2-hour incubation of E. coli with 85 μg/mL of GO or rGO. Transmission electron microscopy (TEM) revealed severe cell membrane damage and cytoplasm leakage, which might be caused by either oxidative stress or physical disruption [43]. Both GO and rGO nanowalls were bactericidal to E. coli as well as Gram-positive Staphylococcus aureus strains when deposited on a stainless steel substrate, with rGO nanowalls being more toxic to both bacteria than the unreduced GO nanowalls. A better charge transfer between bacteria and the sharper edges rGO nanowalls correlated with the severe antibacterial effect. Cell membrane damage caused by direct contact between the bacteria and the extremely sharp edges of the nanowalls is thought to be an effective mechanism for its antibacterial activity [44]. Later, the antimicrobial activity of four types of graphene-based materials (graphite, graphite oxide, GO, and rGO) toward E. coli was studied using the colony counting method. The results indicated that GO had the highest anti-
bacterial activity, followed in decreasing order by rGO, graphite, and graphite oxide [45]. This study proposed a three-step antimicrobial mechanism for GFNs, which was similar to the cytotoxicity mechanism proposed for CNTs. That is, initial cell deposition on GFNs, significant membrane stress caused by direct contact with sharp edges, and subsequent superoxide anion-independent oxidation [45]. Other studies have observed the oxidative stress-mediated antibacterial activity of GO and rGO in additional bacterial strains, e.g., Pseudomonas aeruginosa [46].

Recently, Tu et al. [47] discovered a novel mechanism for grapheme’s cytotoxicity and antibacterial activities, destruc-
tive extraction of phospholipids from E. coli membranes. After a 2.5-hour incubation of E. coli with 100 μg/mL GO nanosheets, three stages of cell damage were observed by TEM. In Stage I (initial morphology), the cells were tolerant to GO for a short period of time; in Stage II, the cell membranes partially lost integrity, with some presenting a lower surface phospholipid density; and in Stage III, the cell membranes were severely damaged and some were even entirely missing their cytoplasm [47]. Subsequent molecular dynamic simulation similarly observed three distinguishable modes thus confirming these results. Firstly, the swing mode: the graphene nanosheet swung back and forth around the restrained atom for tens of nanoseconds. Sec-
dondly, the insertion mode: the edge of the nanosheet entered and cut into the cell membranes in a few nanoseconds due to robust van der Waals attractions from the membrane lipids and hydrophobic interactions. Thirdly, the extraction mode: the nanosheet drew the phospholipid molecules vigorously from the lipid bilayers onto its surface. These observations suggest that both graphene insertion/cutting and destructive lipid extraction lead to serious membrane stress, thus decreasing cell viability [47].

Besides the antibacterial effects of the GFNs, antifungal activity has been reported for rGO nanosheets. A fungal cul-
ture was placed at the center of a Petri dish containing potato dextrose agar and 0–500 μg/mL rGO nanosheets [48]. A 7-day incubation completely inhibited all the mycelia growth at the highest concentration of rGO, and direct contact was proposed as the mechanism for the activity. By contrast, GO exhibited no antifungal effect against Candida albicans or Candida tropicalis. In addition, GO–Ag nanocomposites used as a carrier of silver nanoparticles had prolonged antifungal activity due to the controlled release of silver ions [49]. Moreover, GO–Ag nanocomposites with an optimal ratio of silver nanoparticles to GO also displayed enhanced, strong anti-
bacterial activities against E. coli and S. aureus strains with species-specific mechanisms. GO–Ag nanocomposite dis-
rupted E. coli bacterial wall integrity, whereas it greatly inhibited S. aureus cell division [50].

Recently, ever more graphene-based nanocomposites, such as GO–Ag, GO–TiO$_2$–Ag, poly-l-lysine/rGO/copper nanoparticles, and poly(N-vinylcarbazole)/graphene, have been developed for antimicrobial applications due to their high stability, permeability, and enhanced antimicrobial ac-
tivities [42,50–52]. However, other studies have questioned

4.1. Toxicity in bacteria

Recent studies have investigated GFNs toxicity in both bac-
teria and fungi. Graphene effectively inhibited the growth of Gram-negative Escherichia coli and Gram-positive Bacillus subtilis at a concentration of 1 mg/mL [42]. Two water dispersible graphene derivatives, GO and rGO nanosheets, inhibited the growth of E. coli with minimal cytotoxicity [43]. In the colony-forming assay, more than 90% of the bacteria lost viability following a 2-hour incubation of E. coli with 85 μg/mL of GO or rGO. Transmission electron microscopy (TEM) revealed severe cell membrane damage and cytoplasm leakage, which might be caused by either oxidative stress or physical disruption [43]. Both GO and rGO nanowalls were bactericidal to E. coli as well as Gram-positive Staphylococcus aureus strains when deposited on a stainless steel substrate, with rGO nanowalls being more toxic to both bacteria than the unreduced GO nanowalls. A better charge transfer between bacteria and the sharper edges rGO nanowalls correlated with the severe antibacterial effect. Cell membrane damage caused by direct contact between the bacteria and the extremely sharp edges of the nanowalls is thought to be an effective mechanism for its antibacterial activity [44]. Later, the antimicrobial activity of four types of graphene-based materials (graphite, graphite oxide, GO, and rGO) toward E. coli was studied using the colony counting method. The results indicated that GO had the highest anti-
bacterial activity, followed in decreasing order by rGO, graphite, and graphite oxide [45]. This study proposed a three-step antimicrobial mechanism for GFNs, which was similar to the cytotoxicity mechanism proposed for CNTs. That is, initial cell deposition on GFNs, significant membrane stress caused by direct contact with sharp edges, and subsequent superoxide anion-independent oxidation [45]. Other studies have observed the oxidative stress-mediated antibacterial activity of GO and rGO in additional bacterial strains, e.g., Pseudomonas aeruginosa [46].

Recently, Tu et al. [47] discovered a novel mechanism for grapheme’s cytotoxicity and antibacterial activities, destruc-
tive extraction of phospholipids from E. coli membranes. After a 2.5-hour incubation of E. coli with 100 μg/mL GO nanosheets, three stages of cell damage were observed by TEM. In Stage I (initial morphology), the cells were tolerant to GO for a short period of time; in Stage II, the cell membranes partially lost integrity, with some presenting a lower surface phospholipid density; and in Stage III, the cell membranes were severely damaged and some were even entirely missing their cytoplasm [47]. Subsequent molecular dynamic simulation similarly observed three distinguishable modes thus confirming these results. Firstly, the swing mode: the graphene nanosheet swung back and forth around the restrained atom for tens of nanoseconds. Sec-
dondly, the insertion mode: the edge of the nanosheet entered and cut into the cell membranes in a few nanoseconds due to robust van der Waals attractions from the membrane lipids and hydrophobic interactions. Thirdly, the extraction mode: the nanosheet drew the phospholipid molecules vigorously from the lipid bilayers onto its surface. These observations suggest that both graphene insertion/cutting and destructive lipid extraction lead to serious membrane stress, thus decreasing cell viability [47].

Besides the antibacterial effects of the GFNs, antifungal activity has been reported for rGO nanosheets. A fungal cul-
ture was placed at the center of a Petri dish containing potato dextrose agar and 0–500 μg/mL rGO nanosheets [48]. A 7-day incubation completely inhibited all the mycelia growth at the highest concentration of rGO, and direct contact was proposed as the mechanism for the activity. By contrast, GO exhibited no antifungal effect against Candida albicans or Candida tropicalis. In addition, GO–Ag nanocomposites used as a carrier of silver nanoparticles had prolonged antifungal activity due to the controlled release of silver ions [49]. Moreover, GO–Ag nanocomposites with an optimal ratio of silver nanoparticles to GO also displayed enhanced, strong anti-
bacterial activities against E. coli and S. aureus strains with species-specific mechanisms. GO–Ag nanocomposite dis-
rupted E. coli bacterial wall integrity, whereas it greatly inhibited S. aureus cell division [50].

Recently, ever more graphene-based nanocomposites, such as GO–Ag, GO–TiO$_2$–Ag, poly-l-lysine/rGO/copper nanoparticles, and poly(N-vinylcarbazole)/graphene, have been developed for antimicrobial applications due to their high stability, permeability, and enhanced antimicrobial ac-
tivities [42,50–52]. However, other studies have questioned
the antibacterial and bacteriostatic properties of the GFNs, and suggest GO can act as a scaffold for E. coli bacterial attachment, proliferation, and biofilm formation [53]. These conflicting results encourage additional investigations on the effects of GFNs on microorganisms. However, because GFNs generally have demonstrated bacterial toxicity and relatively low cytotoxicity, it has been suggested that these materials might have applications in antimicrobial products, similar to the most widely used antimicrobial nanoscale substance, silver nanoparticles [54].

4.2. In vitro mammalian cell toxicity

Initial screening of new materials for an in vitro toxicity assessment commonly uses a variety of cell lines. Data from the literature suggest that GFNs exposure may result in cytotoxicity and/or genotoxicity in mammalian cells.

4.2.1. Graphene

A comparative study measuring mitochondrial toxicity and cell membrane integrity in neuronal PC12 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the lactate dehydrogenase (LDH) release assay suggested that the biological activities of graphene and single-wall CNTs (SWCNT) were shape-dependent [55]. After a 24-hour exposure, the metabolic activity of PC12 cells decreased in a dose-dependent manner, with graphene producing higher toxicity at low concentrations and lower toxicity at high concentrations than SWCNT. The highest concentration of graphene in this study (100 µg/mL) significantly increased LDH release and the generation of reactive oxygen species (ROS). In addition, caspase 3 activation indicated that graphene induced a time-dependent increase in apoptosis at a concentration of 10 µg/mL. Yuan et al. [56] compared the potential cytotoxicity of graphene and SWCNT on the human hepatoma HepG2 cell line at the proteome level. These researchers used the isobaric-tagged relative and absolute quantification-coupled two-dimensional liquid chromatography–tandem mass spectrometry (iTRAQ-2D LC-MS/MS) approach to characterize graphene and SWCNT exposed HepG2 cellular functions. Overall, 1 µg/mL of both nanomaterials resulted in differential expression of 37 proteins involved in metabolic pathways, redox regulation, cytoskeleton formation, and cell growth, with graphene resulting in more moderate variations in protein levels. An interesting finding was that graphene and SWCNT produced different patterns in the expression levels of calcium-binding proteins, indicating that they had different modes of action [56].

Later, pristine graphene was also found to increase ROS and apoptosis in murine RAW 264.7 macrophages, an important effector cell of the innate immune system [57,58]. The proposed underlying mechanisms were the depletion of mitochondrial membrane potential (MMP) and ROS-triggered apoptosis by the activation of the mitochondrial pathway. This study found that both mitogen-activated protein kinases and transforming growth factor-β (TGF-β) related signaling pathways were involved in the toxicity of pristine graphene-treated macrophages. The expression of three major phosphorylated kinases (c-Jun N-terminal kinase, p38, and extracellular signal-regulated kinase), two proapoptotic members of the Bcl-2 protein family (Bim and Bax), and TGF-β were significantly upregulated [57].

Human glioblastoma U87 and U118 cells were used to examine the influence of graphene platelets on cell morphology, mortality, viability, membrane integrity, and the type of cell death [9]. Graphene platelets had a strong tendency to localize close to the cells, but not enter into the cells. At a concentration of 100 µg/mL, a 24-hour treatment caused about 50% cell death and loss of membrane integrity and apoptosis. Layered graphene platelets (1–10 layers) at concentrations ≥5 µg/cm² also significantly increased the release of LDH in immortalized human acute monocytic leukemia cells (THP-1), indicating loss of membrane integrity. The depletion of reduced glutathione and higher expression of a panel of cytokines [e.g., monocyte chemotactic protein-1, interleukin (IL)-1, macrophage inflammatory protein-1R, and IL-1β] were also observed after graphene platelet exposure [59].

4.2.2. GO

GO is the most extensively investigated member of GFNs in vitro toxicity studies. Although the first comprehensive study on the toxicity of GO observed neither obvious cellular uptake nor obvious effects on the morphology, viability, mortality, and membrane integrity in adenocarcinomic human alveolar basal epithelial (A549) cells, GO exposure was able to induce oxidative stress at a concentration as low as 10 µg/mL [60]. This, however, is one of the few reports of a negative cytotoxic response for GO in mammalian cells. A few months later, using the same cell line, Hu et al. [61] reported that GO produced concentration-dependent cytotoxicity, which could be largely attenuated by incubation with 10% fetal bovine serum, due to GO’s extremely high protein adsorption ability. Subsequently, the toxicity, genotoxicity, and the potential mechanisms of GO have been reported in a variety of human and animal cell lines, including immortalized and normal cell lines, immune cells, stem cells, and blood components.

In studies using immortalized cells, the toxicity of GO has been reported in the HepG2 cell line. Lammel et al. [62] evaluated the cytotoxicity of 1–16 µg/mL GO by four assays [5-carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM), alamar blue assay, neutral red uptake assay, and fluorescamine assay]. GO caused a dose-dependent decrease in fluorescence intensity starting at 4 µg/mL in the CFDA-AM assay, indicating plasma membrane damage; and loss of plasma membrane structural integrity was associated with a strong physical interaction of GO with the phospholipid bilayer [62]. TEM and scanning electron micrographs demonstrated that GO was able to penetrate through the plasma membrane, resulting in altered cell morphology and an augmented number of apoptotic cells. In addition, the parallel alterations of elevated ROS at concentration as low as 1 µg/mL and dose-related depletion of the MMP suggest that impaired mitochondrial function may lead to intracellular ROS formation. Among the modes of action assessed, the authors concluded that plasma membrane damage and oxidative stress play crucial roles in GO-induced cytotoxicity [62]. Yuan et al. [63] evaluated the cytotoxicity of GO and
oxidized SWCNT in HepG2 cells using iTRAQ-2D LC-MS/MS [66] to characterize cellular function. Similar to their previous study, 1 µg/mL of both GO and oxidized SWCNTs led to altered protein expression involved in metabolic pathways, redox regulation, cytoskeleton formation, and cell growth, with GO inducing much lower changes in expression in comparison to oxidized SWCNTs [63]. Moreover, a minor reduction in proliferation rate, slightly perturbed cell cycle, and elevated intracellular ROS levels also were found in GO-treated cells, suggesting that GO has less cytoxicity in HepG2 cells. The cytoxicities of GO-1 and its repeated KMnO₄–H₂SO₄ oxidation products, GO-2 and GO-3, have been compared in HeLa cells using the MTT assay. The average lateral sizes of GO-1, GO-2, and GO-3 were 205.8 nm, 146.8 nm, and 33.78 nm, respectively [64]. GO-1 produced significant cytotoxicity at concentrations of 20–100 µg/mL, whereas GO-2 and GO-3 exhibited significantly higher viability with higher cellular uptake in HeLa cells, suggesting that the larger sized GO caused greater damage to the cell membrane as compared to the smaller sized GOs. Another study also reported a dose-dependent toxicity of GO in HeLa cells, with a lower cell uptake ratio compared to CNT and nanodiamond [65]. GO cytotoxicity also was reported for human neuroblastoma SH-SYSY cells at concentrations ≥80 µg/mL [66].

GO-induced cytotoxicity, genotoxicity, and oxidative stress have been investigated in normal human lung fibroblast cells [67]. The MTT assay indicated a significant decrease in cell viability and an increase in toxicity following a prolonged treatment time, as well as an apoptotic effect of GO at a concentration of 100 µg/mL. Furthermore, for the first time, the genotoxicity of GO was assessed using the Comet assay. DNA damage, as measured by increased tail length and the percentage of DNA in the tail, were found for all the tested concentrations including 1 µg/mL. It is worth noting that 1 µg/mL GO caused no obvious decrease in cell viability or increase in cellular apoptosis, suggesting that genotoxicity assays may serve as a more sensitive and representative way to detect the toxicity of GO in mammalian cells [67]. The accumulation of ROS, the decreased level of cellular superoxide dismutase, and the reversal in cytotoxicity by the addition of the antioxidant N-acetylcysteine all support an oxidative stress mechanism for GO-induced toxicity in human lung fibroblast cells. A novel finding from this study was that the surface charge of GO and GO derivatives changed their aggregation status as well as their ability to be internalized. It was proposed by the authors that the lower the positive surface charge of GO, the milder the toxic effect of GO on cells. Therefore, it may be useful for clinical applications that new ways are found to attenuate GO toxicity by decreasing its electrical surface charge. Another normal fibroblast cell line, human dermal fibroblast, was cultured with 5–100 µg/mL of GO for 1–5 days [68]. Doses of GO <20 µg/mL had little toxicity to human dermal fibroblast cells, whereas doses ≥50 µg/mL exhibited obvious cytoxicity, such as decreased cell survival by cell counts, reduced adhesive ability, and increased cell apoptosis. TEM revealed that GO was indeed internalized by the cells and subcellularly localized to the lysosomes, mitochondria, endoplasm, and even the cell nucleus in a time- and dose-dependent manner [68].

GO toxicity also has been described in BEAS-2B human lung cells and the HBI.F3 human neural stem cell line. In BEAS-2B cells, significant concentration- and time-dependent decreases in cell viability were observed at concentrations of 10–100 µg/mL by the MTT assay, and both early and late apoptotic cells were increased when compared to the control [69]. HBI.F3 cell viability was decreased with increasing GO nanopellet concentration (25–200 µg/mL), which was verified by both the MTT assay and differential pulse voltammetry, a microscopic imaging tool [70].

In comparison to previous studies, GO had minimal toxicity in spontaneously arising human retinal pigment epithelium (ARPE-19) cells. Toxicity was measured by cell morphology, viability, membrane integrity, and apoptosis using various approaches, including optical micrography, the CCK-8 assay, LDH assay, and apoptosis assay [71]. The cells were found to be in a good condition after the addition of up to 100 µg/mL GO for 72 hours, but the cell morphology exhibited some alterations following 7 days’ culture with GO. At all concentrations, <8% of cells released LDH, indicating little damage to the cell membrane. This study suggests that GO has good biocompatibility with retinal pigment epithelium cells, producing only slight effects on cell viability and morphology [71].

The ability of GO to induce immunotoxicity and potential mechanisms for this toxicity have also been studied [72–74]. Three types of immune cells, macrophages, dendritic cells, and T-lymphocytes, were isolated from healthy donor blood and treated with up to 100 µg/mL of GO. The levels of three cytokines (tumor necrosis factor-α, IL-1β, and IL-6), measured by the enzyme-linked immunosorbent assay, were increased in dendritic cells with increasing GO doses. Exposure to GO also induced apoptosis in T-lymphocytes in a dose-dependent manner as measured by the Annexin V+/PI+ assay. Macrophages easily ingested GO and formed black dense aggregates within the cells; GO coated with polyvinylpyrrolidone exhibited improved immunological biocompatibility in vitro [73]. Chen et al. [74] found that treatment of RAW264.7 macrophages with GO resulted in autophagic vacuoles and activation of autophagic marker proteins. Molecular analysis demonstrated the toll-like receptor (TLR) signaling cascades and the ensuing cytokine pathway were involved in the GO-induced inflammatory response [74]. The evidence indicated that interaction of GO with TLR4 was probably responsible for GO-induced macrophage necrosis. Inhibition of the TLR4 signaling with a selective TLR4 inhibitor (CLI-095) greatly reduced GO-induced cell death [72].

As GFNs may enter blood during drug delivery applications, it is important to assess their haemocompatibility and toxicity to blood components. Liao et al. [75] compared the cytoxicity of graphene sheets and GO in human erythrocytes and CRL-2522 adherent human skin fibroblasts. The results indicated that the graphene and GO toxicity was environment-dependent, e.g., whether aggregation occurred and the mode of interaction with cells. By measuring the efflux of hemoglobin from suspended red blood cells, the authors observed that the smallest sized GO had the greatest hemolytic activity, whereas aggregated graphene exhibited the lowest hemolytic activity. Similarly, compacted graphene sheets induced more...
damage to skin fibroblasts than the less densely packed GO as measured by the water-soluble tetrazolium salt, trypan blue exclusion, and ROS assays [75]. Moreover, this study suggested that the MTT assay might lead to a false-positive result by overestimating cell viability. This is because GO reacts with the MTT reagent and forms purple formazan. In blood platelets, atom-thin GO sheets elicited a strong aggregate response to the MTT reagent and forms purple formazan. In blood platelets, atom-thin GO sheets elicited a strong aggregate response through the activation of Src kinases and the release of calcium from intracellular stores [76].

4.2.3. rGO

In an early rGO study with three cell types (PC12 cells, oligodendroglia cells, and osteoblasts), rGO films were found to be more biocompatible as compared to SWCNT [77]. Recently, the size- and concentration-dependent cytotoxicity and genotoxicity of rGO and GO nanoplatelets has been studied in fresh human mesenchymal stem cells (hMSCs) isolated from umbilical cord blood. Toxicity was measured using the fluorescein diacetate cell viability assay, RNA efflux, and the Comet and chromosomal aberration assays [78]. The fluorescein diacetate test showed significant cytotoxic effects for rGO with an average lateral dimension of 11 nm (the smallest rGO in this study), even at the lowest concentration of 1 µg/mL and after a 1-hour exposure. rGOs with an average lateral dimension of 3.8 µm, the largest-sized-rGO used in this study, exhibited lower cytotoxicity as compared to rGOs with average lateral dimensions of 91 nm and 418 nm [78]. Assays for RNA efflux from cells, an indirect indicator of membrane damage, determined there was a consistent size- and concentration-dependent response in rGO-treated hMSCs. The smaller-sized-rGO induced higher RNA effluxes than did the larger-sized-rGO sheets. Moreover, rGOs generated 13–26-fold higher levels of ROS when compared to the control. This suggests that oxidative stress is one of the mechanisms involved in rGO cytotoxicity. In the genotoxicity study, 1-hour’s exposure to rGOs with average lateral dimensions of 11 nm and 91 nm initiated significant increases in DNA damage and chromosomal aberration frequency at concentrations as low as 0.1 µg/mL and 1.0 µg/mL, respectively. These concentrations were 10 times lower than the threshold concentration observed in the cell viability test. The two larger-sized rGO sheets induced only slight DNA fragmentation at the highest concentration of 100 µg/mL and after a longer exposure time of 24 hours [78]. These results suggest that the interaction of rGOs with hMSCs, and probably other cells, strongly depends on their lateral size. The most likely mechanisms involved in rGO cytotoxicity are oxidative stress and direct contact of the sharp edges with the cells. The latter may subsequently induce genotoxicity in cells through interaction of the penetrated nanosheets with the nucleus of the cells [78].

rGO induced significantly higher cytotoxicity than GO in A549 cells. Using the MTT assay, Hu et al. [43] found that rGO nanosheets with a thickness of 4.6 Å reduced cell viability to 47% and 15% at concentrations of 20 µg/mL and 85 µg/mL, respectively. Green synthesized rGO, namely bacterially rGO, also induced higher levels of cytotoxicity, ROS, and loss of membrane integrity in MCF-7 cells as compared to GO [79]. Incubation of MCF-7 cells with both bacterially rGO or GO at doses above 60 µg/mL produced marked cytotoxic effects, including decreased cell viability, increased ROS generation, and release of LDH [79].

4.2.4. Functionalized graphene-family nanomaterials

Most GFNs tend to aggregate in physiological solutions due to electrostatic charges and nonspecific binding to protein [19]. Therefore, development of functionalized GFNs has improved their solubility and biocompatibility, and reduced cytotoxicity and genotoxicity. Two main strategies, covalent conjugation and noncovalent physiosorption, are commonly used for surface modification of GFNs to build desired functionalized GFNs [19,26]. Polymers or molecules used for covalent GFN modification include many types of aliphatic and aromatic amines, amino acids, amine terminated biomolecules, silanes, and enzymes [26]. Examples of GFN modifiers include PEG [29], polyethylenimine (PEI) [80], polyvinyl alcohol [81], and chitosan [82,83]. Noncovalent methods of functionalization employ hydrophobic interaction, π–π interaction, van der Waals forces, and electrostatic binding [19,26], and they appear to be more versatile than covalent methods. Reports of both covalent conjugation and noncovalent physiosorption techniques indicate distinct decreases in GFN’s toxic side effects.

Sasidharan et al. [58,84] compared interactions of pristine graphene and functionalized graphene with monkey renal epithelial cells, murine RAW 264.7 macrophages, and primary human blood components. In the monkey cells, internalization of functionalized graphene into the cells did not produce any obvious short-term toxicity, whereas pristine graphene accumulated on the cell membrane leading to ROS-mediated apoptosis [84]. Similar to this observation, pristine graphene was mainly retained on the surface of RAW 264.7 macrophages, resulting in significantly reduced cell viability and the formation of intracellular ROS in ~24.2% of cells at a concentration of 75 µg/mL. By contrast, only 4% of functionalized graphene-treated cells exhibited ROS generation with no toxic effect at concentrations of up to 75 µg/mL and even with very high intracellular uptake. Furthermore, treatment of peripheral blood mononuclear cells with pristine graphene produced higher expression of IL-8 and IL-6 than treatment with functionalized graphene, indicating a higher inflammatory potential for pristine graphene. These results suggest that surface functionalization of pristine graphene can prevent much of its toxicity [58].

To determine the effects of functionalized GFNs on blood component toxicity, the effect of amine-modified graphene on platelet reactivity has been evaluated [85]. Unlike GO and rGO, which prompted a strong aggregatory response in platelets, amine-modified graphene had no stimulatory effect on human platelets. Intravenous administration of amine-modified graphene also did not potentiate the lysis of erythrocytes or pulmonary thromboembolism in mice. These results indicate that amine-modified graphene is potentially safe for in vivo biomedical applications [85]. Comparison of GO and PEGylated nano-GO interactions with human serum components revealed that GO adsorbed numerous serum proteins and strongly induced complement C3 cleavage thus forming C3a/C3a(des-Arg), an anaphylatoxin involved in local inflammatory responses. By comparison, PEGylated GO had significantly lower levels of both serum protein binding and complement C3 activation [86]. Interestingly, PEGylated nano-
GO also selectively increased the binding capacities of six serum proteins, and four of them were immune-related factors, including C3a/C3a(des-Arg). These findings suggest that PEGylated nano-GO may serve as an immune response modulator to eliminate C3a/C3a(des-Arg) resulting from other nanomaterials [86].

Functionalization, however, does not always eliminate the toxicity of GFNs. The reported IC50 of noncovalent PEGylated nanosized GO was about 80–85 μg/mL in both the human breast cancer cell line MCF-7 and human glioblastoma U87MG cells [87]. Carboxyl graphene, having a high carboxyl ratio and additional ethanoic acid groups (–O–CH2–COOH) on sp2-hybridized carbon on the basal plane, was found to cause a dose-dependent decrease in fluorescence intensity starting from 4 μg/mL in the CFDA-AM assay, indicating plasma membrane damage. In addition, carboxyl graphene increased ROS production and produced a dose-related depletion of MMP in HepG2 cells [62].

The adverse effects of GO nanoribbons water-solubilized with PEG-DsPE (1,2-distearyl-sn-glycero-3-phosphate-N-amino-PEG; O-GNR-PEG-DsPE) have been assessed using different toxicity assays (including the alamar blue, neutral red, LDH release, trypan blue, and clonogenic assays) in four representative cell lines [HeLa, MCF-7, NIH 3T3 mouse fibroblasts, and Sloan Kettering breast cancer cells (SKBR3)] [88]. Test doses ranged from 10 μg/mL to 400 μg/mL, and all cells experienced dose- and time-dependent reductions in cell viability. HeLa cells displayed the highest cellular uptake and cytotoxicity as determined by the cellular metabolism, lysosomal integrity, LDH release, and cell proliferation assays. Significant cell death (5–25%, depending on the time point and the assay) for HeLa cells occurred at concentrations >10 μg/mL. By contrast, about 78% of cells were still viable at the highest concentration of 400 μg/mL in SKBR3 and MCF-7 cells, indicating that water-solubilized O-GNR-PEG-DsPE has a heterogenous cell-specific cytotoxicity [88].

The cytotoxicity of three derivatives of GO, PEI-GO, PEG-GO, and lactobionic acid-polyethylene glycol (LA-PEG) functionalized GO (LA-PEG-GO), was compared in human lung fibroblast cells: the order of cytotoxicity was PEG-GO < LA-PEG-GO < GO < PEI-GO [67]. The Comet assay indicated that PEG and LA-PEG modified GOs were less genotoxic, and that PEI-modified GO damaged DNA to a similar extent as unmodified GO.

4.3. In vivo toxicity

Information on the in vivo toxicity of GFNs is essential if they are to be used for drug delivery. The in vivo toxicity of GO has been studied in Kunming mice [68]. No toxicity was detected in mice exposed intravenously to GO at a low (0.1 mg) and middle (0.25 mg) dose, whereas a high dose of GO (0.4 mg) resulted in chronic toxicity. Four of nine mice died from suffocation 1–7 days after injection due to blockage of the major airways by GO conglomeration. GO accumulation was detected primarily in the lungs, liver, and spleen. For the surviving mice, obvious chronic toxicity occurred mainly in the lungs and liver. Histopathological analysis revealed a dose-dependent lung inflammatory response characterized by neutrophils and foamy alveolar macrophage accumulation and epithelioid granulomas formation. The accumulation of GO in liver indicated that GO might mainly be eliminated by liver secretion into the bile tract system, because little GO was observed in the kidney. These results suggest that GO may not be suitable for human use because its shape makes it very difficult for the kidney to remove [68]. A similar study in mice also demonstrated that GO was cleared from the blood quickly and accumulated mainly in the liver and lungs, with the larger sizes of GO (1–5 μm) accumulating in the lungs whereas smaller sizes (110–500 nm) were retained by the liver [89]. In addition, intratracheal injection of 50 μg GO in C57BL/6 mice induced severe and persistent injuries in the lungs. Dispersion of pristine graphene with the blocking copolymer Pluronic greatly reduced the toxicity [90]. Another study also demonstrated extensive pulmonary thromboembolism in Swiss male mice only 15 minutes after intravenously administrating 250 μg/kg body weight GO [76]. However, GO purification via several washings may eliminate the toxicological and inflammatory effects as no inflammation or granuloma formation was induced in female C57BL/6 mice following intraperitoneal injection of 50 μg highly pure, colloidally stable, and evenly dispersed GO in physiologically relevant aqueous buffers [24,91].

In addition to pulmonary inflammation and thromboembolism, immune responses were detected in the lungs of C57BL/6 mice after intravenous administration of 1 mg/kg body weight of graphene nanosheets [92]. Graphene nanosheets triggered an increase in interleukin (IL)-33 and its soluble receptor sST2 in the bronchoalveolar lavage fluid 1 day following injection, and resulted in a Th2 immune response consisting of neutrophilic influx and increases in IL-5 and IL-13. Results using ST2−/− mice indicated that the site-specific Th2 immune responses of graphene nanosheets were dependent upon the IL-33/ST2 axis.

To investigate the potential adverse effects of GO on the eye, Japanese white rabbits were injected intravitreally with 0.1 mg, 0.2 mg, or 0.3 mg of GO and monitored for up to 49 days. There was no clinical evidence of forocarial changes and GO had a negligible influence on both the intraocular pressure and eyesight in treated animals as determined by slit lamp biomicroscopy and indirect funduscopy examination [71]. GO content decreased gradually in the eyes during the observation period, and histological examination at the conclusion of the experiment observed a very small amount of residual GO with no retinal abnormality in the GO-injected eyes. These results suggest that intravitreal injection of up to 0.3 mg of GO has no significant negative effects on the eyes [71].

Surface modifications can also modulate the toxicity of graphene in vivo. A series of the in vivo pharmacokinetics, biodistribution, and toxicity studies of graphene and PEGylated graphene using three administration routes (intravenous, intraperitoneal, and oral) have been performed in Balb/c mice [25,93,94]. One hour after an intravenous injection dose of 20 mg/kg, PEG-graphene nanosheets were distributed in many different organs. Three days later, PEG-graphene was found mainly in the reticuloendothelial system, including the spleen and liver [94]. Toxicity studies of PEG-graphene nanosheets revealed neither death nor significant body weight drop in the mice during the 90-day treatments. Blood biochemistry and hematology analysis did not
detect any changes in the liver and kidney functional markers including alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. The ratio of albumin and globulin, the urea levels in the blood, and all hematological markers were also unchanged [94]. In addition, there was no obvious organ damage except for a brown discoloration in the liver and spleen due to the accumulation of PEG-graphene nanosheets in the 1st 20 days [94]. Recently, Yang et al. [93] investigated the biodistribution and potential toxicity of GO and several PEGylated GO derivatives with different sizes and surface coatings after oral and intraperitoneal administrations to BALB/c mice at a dose of 4 mg/kg [93]. No obvious tissue uptake was observed following oral administration, indicating limited intestinal adsorption of these nanomaterials. By contrast, following intraperitoneal injection, the researchers detected a much higher accumulation of PEG-GO derivatives, but not GO, in the reticuloendothelial system, including the liver and spleen. Similar to their previous study, histological examination of organ sections and hematological analysis revealed only insignificant changes to animals, although the nanomaterials did persist in the mouse body over 3 months [93]. These results suggest that the in vivo behavior and toxicity of GFNs depend on the administration route.

Inhalation toxicity is a major concern for the production and use of nanomaterials. Due to their similar aerodynamic properties, the inhalation toxicity of graphene nanoplatelets (average diameter of 5 μm) and nanoparticulate carbon black (diameter of 10 nm) has been studied in C57BL/6 mice [59]. No obvious tissue uptake was observed following oral administration, indicating limited intestinal adsorption of these nanomaterials. By contrast, following intraperitoneal injection, the researchers detected a much higher accumulation of PEG-GO derivatives, but not GO, in the reticuloendothelial system, including the liver and spleen. Similar to their previous study, histological examination of organ sections and hematological analysis revealed only insignificant changes to animals, although the nanomaterials did persist in the mouse body over 3 months [93]. These results suggest that the in vivo behavior and toxicity of GFNs depend on the administration route.

Inhalation toxicity is a major concern for the production and use of nanomaterials. Due to their similar aerodynamic properties, the inhalation toxicity of graphene nanoplatelets (average diameter of 5 μm) and nanoparticulate carbon black (diameter of 10 nm) has been studied in C57BL/6 mice [59]. No obvious tissue uptake was observed following oral administration, indicating limited intestinal adsorption of these nanomaterials. By contrast, following intraperitoneal injection, the researchers detected a much higher accumulation of PEG-GO derivatives, but not GO, in the reticuloendothelial system, including the liver and spleen. Similar to their previous study, histological examination of organ sections and hematological analysis revealed only insignificant changes to animals, although the nanomaterials did persist in the mouse body over 3 months [93]. These results suggest that the in vivo behavior and toxicity of GFNs depend on the administration route.

Inhalation toxicity is a major concern for the production and use of nanomaterials. Due to their similar aerodynamic properties, the inhalation toxicity of graphene nanoplatelets (average diameter of 5 μm) and nanoparticulate carbon black (diameter of 10 nm) has been studied in C57BL/6 mice [59]. No obvious tissue uptake was observed following oral administration, indicating limited intestinal adsorption of these nanomaterials. By contrast, following intraperitoneal injection, the researchers detected a much higher accumulation of PEG-GO derivatives, but not GO, in the reticuloendothelial system, including the liver and spleen. Similar to their previous study, histological examination of organ sections and hematological analysis revealed only insignificant changes to animals, although the nanomaterials did persist in the mouse body over 3 months [93]. These results suggest that the in vivo behavior and toxicity of GFNs depend on the administration route.

Further in vivo toxicological studies exposed Caenorhabditis elegans to GFNs [96–98]. C. elegans is a free-living, transparent nematode with a length of about 1 mm. Exposure to 250 μg/mL graphite nanoplatelets, consisting of 3–60 graphene layers with a lateral size of 1–10 μm, resulted in no detectable toxicity as measured by longevity and reproductive capacity [97]. By contrast, prolonged oral administration at doses of 0.5–100 μg/mL GO damaged both primary (intestine) and secondary (nerve and reproductive organ) targeted organs, and the change was closely correlated with ROS production [96]. GO was translocated into intestinal cells with the loss of microvilli and distributed to surrounding mitochondria. GO also caused a prolonged defecation cycle and alterations in the expression of genes responsible for intestinal development and defection behavior. These results indicate that long-term exposure to GO may place environmental organisms at risk due to the combinational effects of oxidative stress, enhanced permeability of the biological barrier, and prolonged defection behavior [96].

A dual-path chemical mechanism was developed for the C. elegans model, involving the overproduction of hydroxyl radicals and the formation of oxidizing cytochrome c intermediates, to account for GO’s toxic properties under both normal and stress conditions [98]. Under normal conditions, 10 μg/mL and 20 μg/mL of PEGylated poly-L-lysine GO triggered a moderate ROS elevation but had no influence on worm behavior or reproductive ability. When the worms were placed under conditions of oxidative stress and heat stress, exposure to 5 μg/mL and 20 μg/mL PEGylated poly-L-lysine GO significantly decreased nematode lifespan. When exposed to stress, the cytochrome complex could be translocated from the mitochondria to the cytoplasm, providing an opportunity for direct contact with PEGylated poly-L-lysine GO [98]. Thus, under pathophysiological conditions, GO-included toxicity may involve the cytochrome c/H2O2 systems.

5. Summary

The applications of GFNs have developed rapidly in the past few years. Thus, a comprehensive understanding of the interaction of GFNs with living systems and their adverse effects in vitro and in vivo are essential for further development and safe use of graphene-based nanomaterials.

The majority of current literature agree that unmodified graphene, GO, and rGO are cytotoxic and/or genotoxic. Although surface modified GFNs with ultra-small sizes, excellent dispersibility, and stability in physiological environments are often less toxic, there are inconsistencies between studies. Dose is one of the most important factors and some researchers believe that low doses of GFNs may be safe; sometimes they can even serve as enhancers of cell
proliferation [53]. Further, the physicochemical properties of GFNs, such as the particle size, particulate state, surface functional groups, and oxygen content/surface charges may significantly affect their toxicity in biological systems. The Organization for Economic Co-operation and Development has concluded that the current testing approaches are generally acceptable for nanomaterials, although modifications may be necessary for some test guidelines. The current results indicate that genotoxicity assays may serve as a sensitive approach for evaluating the adverse effects of GFNs, and there is a demand for more data on genotoxicity testing, because only a few published studies are in the literature. Moreover, the presence of contaminants during the processing of nanomaterials may also contribute to their adverse effects. Although the mechanisms for their toxicity have not been determined definitively, ROS is the most widely recognized mechanism for GFN-induced toxicity in living systems.

It is difficult to compare the toxicological effects of GFNs between different studies due to the diversity in the sizes, shapes, surfaces, and the fabrication of GFNs. For example, different production methods cause different amounts of oxygen to be bound to the surface of GFNs, which has proven to be correlated with their toxicity towards cells and other living systems [99]. There is a need for standardizing the terminology, the fabrication of GFNs, and the validation of toxicological methodologies. Standardization will provide necessary information to researchers for better understanding the physicochemical characteristics and the potential toxicological effects in cells and animals, thus facilitating the practical applications of these promising new nanomaterials in humans.

**Conflicts of interest**

All contributing authors declare no conflicts of interest.

**Acknowledgments**

We thank Drs Robert Heflich, Dayton Petibone, and Meagan Myers for their helpful suggestions and comments.

**References**

[1] FDA. Nanotechnology task force report 2007. USFDA; 2007. http://www.fda.gov/downloads/ScienceResearch/SpecialTopics/Nanotechnology/ucm110856.pdf [accessed 04.02.14].
[2] Nel A, Xia T, Mäder L, et al. Toxic potential of materials at the nanolevel. Science 2006;311:622–7.
[3] Xia T, Li N, Nel AE. Potential health impact of nanoparticles. Annu Rev Public Health 2009;30:137–50.
[4] Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 2005;113:823–39.
[5] Novoselov KS, Geim AK, Morozov SV, et al. Electric field effect in atomically thin carbon films. Science 2004;306:666–9.
[6] Geim AK, Novoselov KS. The rise of graphene. Nat Mater 2007;6:183–91.
[7] Li D, Müller MB, Gilje S, et al. Processable aqueous dispersions of graphene nanosheets. Nat Nanotechnol 2008;3:101–5.
[8] Stankovich S, Dikin DA, Dommett GH, et al. Graphene-based composite materials. Nature 2006;442:282–6.
[9] Jaworski S, Sawosz E, Grodzik M, et al. In vitro evaluation of the effects of graphene platelets on glioblastoma multiforme cells. Int J Nanomedicine 2013;8:413–20.
[10] Yang K, Li Y, Tan X, et al. Behavior and toxicity of graphene and its functionalized derivatives in biological systems. Small 2012;9:1492–503.
[11] Singh N, Srivastava G, Talat M, et al. Cicer alphagalactosidase immobilization onto functionalized graphene nanosheets using response surface method and its characterization. Food Chem 2014;142:430–8.
[12] Dikin DA, Stankovich S, Zimney EJ, et al. Preparation and characterization of graphene oxide paper. Nature 2007;448:457–60.
[13] Li N, Chen Z, Ren W, et al. Flexible graphene-based lithium ion batteries with ultrafast charge and discharge rates. Proc Natl Acad Sci USA 2012;109:17360–5.
[14] Zhang L, Xia J, Zhao Q, et al. Functional graphene oxide as a nanocarrier for controlled loading and targeted delivery of mixed anticancer drugs. Small 2010;6:537–44.
[15] Zhu Z, Garcia-Gancedo L, Flewitt AJ, et al. A critical review of glucose biosensors based on carbon nanomaterials: carbon nanotubes and graphene. Sensors (Basel) 2012;12:5996–6022.
[16] Sanchez VC, Jackah A, Hurt RH, et al. Biological interactions of graphene-family nanomaterials: an interdisciplinary review. Chem Res Toxicol 2012;25:15–34.
[17] An J, Gou Y, Yang C, et al. Synthesis of a biocompatible gelatin functionalized graphene nanosheets and its application for drug delivery. Mater Sci Eng C Mater Biol Appl 2013;33:2827–37.
[18] Liu Y, Yu D, Zeng C, et al. Biocompatible graphene oxide-based glucose biosensors. Langmuir 2010;26:6158–60.
[19] Pan Y, Sahoo NG, Li L. The application of graphene oxide in drug delivery. Expert Opin Drug Deliv 2012;9:1365–76.
[20] Ahmed F, Rodrigues DF. Investigation of acute effects of graphene oxide on wastewater microbial community: a case study. J Hazard Mater 2013;256:7:33–9.
[21] Li Y, Yuan H, von dem Bussche A, et al. Graphene microsheets enter cells through spontaneous membrane penetration at edge asperities and corner sites. Proc Natl Acad Sci USA 2013;110:12295–300.
[22] Yue H, Wei W, Yue Z, et al. The role of the lateral dimension of graphene oxide in the regulation of cellular responses. Biomaterials 2012;33:4013–21.
[23] Truong QT, Pokharel P, Song GS, et al. Preparation and characterization of graphene nanoplatelets from natural graphite via intercalation and exfoliation with tetraalkylammoniumbromide. J Nanosci Nanotechnol 2012;12:4305–8.
[24] Ali-Boucetta H, Bitounis D, Raveendran-Nair R, et al. Purified graphene oxide dispersions lack in vitro cytotoxicity and in vivo pathogenicity. Adv Healthc Mater 2013;2:433–41.
[25] Yang K, Zhang S, Zhang G, et al. Graphene in mice: ultrahigh in vivo tumor uptake and efficient photothermal therapy. Nano Lett 2010;10:3318–23.
[26] Liu J, Cui L, Losic D. Graphene and graphene oxide as new nanocarriers for drug delivery applications. Acta Biomater 2013;9:9243–57.
[27] Yang K, Feng L, Shi X, et al. Nano-graphene in biomedicine: theranostic applications. Chem Soc Rev 2013;42:530–47.
[28] Sun X, Liu Z, Welsher K, et al. Nano-graphene oxide for cellular imaging and drug delivery. Nano Res 2008;1:203–12.
[29] Liu Z, Robinson JT, Sun X, et al. PEGLyated nanographene oxide for delivery of water-insoluble cancer drugs. J Am Chem Soc 2008;130:10876–7.
[30] Hu H, Yu J, Li Y, et al. Engineering of a novel pluronic F127/graphene nanohybrid for pH responsive drug delivery. J Biomed Mater Res A 2012;100:141–8.
[31] Qin XC, Guo ZY, Liu ZM, et al. Folic acid-conjugated graphene oxide for cancer targeted chemo-photothermal therapy. J Photochem Photobiol B 2013;120:156–62.
[32] Wei G, Yan M, Dong R, et al. Covalent modification of reduced graphene oxide by means of diazonium chemistry and use as a drug-delivery system. Chemistry 2012;18:14708–16.
[33] Liu G, Shen H, Mao J, et al. Transferrin modified graphene oxide for glioma-targeted drug delivery: in vitro and in vivo evaluations. ACS Appl Mater Interfaces 2013;5:6909–14.
[34] Wang Y, Wang K, Zhao J, et al. Multifunctional mesoporous silica-coated graphene nanosheet used for chemo-photothermal synergistic targeted therapy of glioma. J Am Chem Soc 2013;135:4799–804.
[35] Wang Z, Zhou C, Xia J, et al. Fabrication and characterization of a triple functionalization of graphene oxide with Fe3O4, folic acid and doxorubicin as dual-targeted drug nanocarrier. Colloids Surf B Biointerfaces 2013;106:60–5.
[36] Miao W, Shim G, Kang CM, et al. Cholesteryl hyaluronic acid-coated, reduced graphene oxide nanosheets for anti-cancer drug delivery. Biomaterials 2013;34:9638–47.
[37] Liu CW, Xiong F, Jia HZ, et al. Graphene-based anticancer nanosystem and its biosafety evaluation using a zebrafish model. Biomacromolecules 2013;14:358–66.
[38] Arora A, Padua GW. Review: nanocomposites in food packaging. J Food Sci 2010;75:843–9.
[39] Kumar S, Ghosh S, Munichandariah N, et al. 1.5 V battery driven reduced graphene oxide-silica nanostructure coated carbon foam (rGO-Ag-CF) for the purification of drinking water. Nanotechnology 2013;24:235101.
[40] Li YT, Qu LL, Li DW, et al. Rapid and sensitive in-situ detection of polar antibiotics in water using a disposable Ag-graphene sensor based on electrophoretic preconcentration and surface-enhanced Raman spectroscopy. Biosens Bioelectron 2013;43:94–100.
[41] Jian JM, Liu YY, Zhang YL, et al. Fast and sensitive detection of Pb2+ in foods using disposable screen-printed electrode modified by reduced graphene oxide. Sensors (Basel) 2013;13:15063–75.
[42] Santos CM, Mangadla J, Ahmed F, et al. Graphene nanocomposite for biomedical applications: fabrication, antimicrobial and cytotoxic investigations. Nanotechnology 2012;23:395101.
[43] Hu W, Peng C, Luo W, et al. Graphene-based antibacterial paper. ACS Nano 2010;4:4317–23.
[44] Akhavan O, Ghaderi E. Toxicity of graphene and graphene oxide nanowalls against bacteria. ACS Nano 2010;4:5731–6.
[45] Liu S, Zeng TH, Hofmann M, et al. Antibacterial activity of graphite, graphene oxide, graphene oxide, and reduced graphene oxide: membrane and oxidative stress. ACS Nano 2011;5:6971–80.
[46] Gurunathan S, Han JW, Dayem AA, et al. Oxidative stress-mediated antibacterial activity of graphene oxide and reduced graphene oxide in Pseudomonas aeruginosa. Int J Nanomedicine 2012;7:5901–14.
[47] Tu Y, Lv M, Xiu P, et al. Destructive extraction of phospholipids from Escherichia coli membranes by graphene nanosheets. Nat Nanotechnol 2013;8:594–601.
[48] Sawangphruk MS, Srimuka P, Chiochan P, et al. Synthesis and antifungal activity of reduced graphene oxide nanosheets. Carbon 2012;50:1556–61.
[49] Li C, Wang X, Chen F, et al. The antifungal activity of graphene oxide-silver nanocomposites. Biomaterials 2013;34:3882–90.
[50] Tang J, Chen Q, Xu L, et al. Graphene oxide-silver nanocomposite as a highly effective antibacterial agent with species-specific mechanisms. ACS Appl Mater Interfaces 2013;5:3867–74.
[51] Liu L, Bai H, Liu J, et al. Multifunctional graphene oxide-TiO2-Ag nanocomposites for high performance water disinfection and decontamination under solar irradiation. J Hazard Mater 2013;261:214–23.
[52] Ouyang Y, Cai X, Shi Q, et al. Poly-1-lysine-modified reduced graphene oxide stabilizes the copper nanoparticles with higher water-solubility and long-term additively antibacterial activity. Colloids Surf B Biointerfaces 2013;107:107–14.
[53] Ruiz ON, Fernando KA, Wang B, et al. Graphene oxide: a nonspecific enhancer of cellular growth. ACS Nano 2011;5:8100–7.
[54] Chaloupka K, Malam Y, Seifalian AM. Nanosilver as a new generation of nanoproduct in biomedical applications. Trends Biotechnol 2010;28:580–8.
[55] Zhang Y, Ali SF, Dervishi E, et al. Cytotoxicity effects of graphene and single-wall carbon nanotubes in neural phaeochromocytoma-derived PC12 cells. ACS Nano 2010;4:3181–6.
[56] Yuan J, Gao H, Ching CB. Comparative protein profile of human hepatoma HepG2 cells treated with graphene and single-walled carbon nanotubes: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. Toxicol Lett 2011;207:213–21.
[57] Li Y, Liu Y, Fu Y, et al. The triggering of apoptosis in macrophages by pristine graphene through the MAPK and TGF-beta signaling pathways. Biomaterials 2012;33:402–11.
[58] Sasidharan A, Panchakarla LS, Sadanandan AR, et al. Hemocompatibility and macrophage response of pristine and functionalized graphene. Small 2012;8:1251–63.
[59] Schindwald A, Murphy FA, Jones A, et al. Graphene-based nanoplatelets: a new risk to the respiratory system as a consequence of their unusual aerodynamic properties. ACS Nano 2012;6:736–46.
[60] Chang Y, Yang ST, Liu JH, et al. In vitro toxicity evaluation of graphene oxide on A549 cells. Toxicol Lett 2011;200:201–10.
[61] Hu W, Peng C, Lv M, et al. Protein corona-mediated mitigation of cytotoxicity of graphene oxide. ACS Nano 2011;5:3693–700.
[62] Lammel T, Boisseaux P, Fernández-Cruz ML, et al. Internalization and cytotoxicity of graphene oxide and carboxyl graphene nanoplatelets in the human hepatocellular carcinoma cell line Hep G2. Part Fibre Toxicol 2013;10:27.
[63] Yuan J, Gao H, Sui J, et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. Toxicol Sci 2012;126:149–61.
[64] Zhang H, Peng C, Yang J, et al. Uniform ultrasmall graphene oxide nanosheets with low cytotoxicity and high cellular uptake. ACS Appl Mater Interfaces 2013;5:1761–7.
[65] Zhang X, Hu W, Li J, et al. A comparative study of cellular uptake and cytotoxicity of multi-walled carbon nanotubes, graphene oxide, and nanodiamond. Toxicol Res 2012;2:62–8.
[66] Lv M, Zhang Y, Liang L, et al. Effect of graphene oxide on undifferentiated and retinoic acid-differentiated SH-SY5Y cells line. Nanoscale 2012;4:861–6.
[67] Wang A, Pu K, Dong B, et al. Role of surface charge and oxidative stress in cytotoxicity and genotoxicity of graphene
oxide towards human lung fibroblast cells. J Appl Toxicol 2013;33:1156–64.

[68] Wang K, Ruan J, Song H, et al. Biocompatibility of graphene oxide. Nanoscale Res Lett 2011;6:8–15.

[69] Vallabani NV, Mittal S, Shukla RK, et al. Toxicity of graphene in normal human lung cells (BEAS-2B). J Biomed Nanotechnol 2011;7:106–7.

[70] Kang SM, Kim TH, Choi JW. Cell chip to detect effects of graphene oxide nanopellet on human neural stem cell. J Nanosci Nanotechnol 2012;12:5185–90.

[71] Yan L, Wang Y, Xu X, et al. Can graphene oxide cause damage to eyesight? Chem Res Toxicol 2012;25:1265–70.

[72] Qu G, Liu S, Zhang S, et al. Graphene oxide induces toll-like receptor 4 (TLR4)-dependent necrosis in macrophages. ACS Nano 2013;7:5732–45.

[73] Zhi X, Fang H, Bao C, et al. The immunotoxicity of graphene oxides and the effect of PVP-coating. Biomaterials 2013;34:5254–61.

[74] Chen GY, Yang HJ, Lu CH, et al. Simultaneous induction of autophagy and toll-like receptor signaling pathways by graphene oxide. Biomaterials 2012;33:6559–69.

[75] Liao KH, Lin YS, Macosko CW, et al. Cytotoxicity of graphene oxide and graphene in human erythrocytes and skin fibroblasts. ACS Appl Mater Interfaces 2011;3:2607–15.

[76] Singh SK, Singh MK, Nayak MK, et al. Thrombus inducing property of atomically thin graphene oxide sheets. ACS Nano 2011;5:4987–96.

[77] Agarwal S, Zhou X, Ye F, et al. Interfacing live cells with nanocarbon substrates. Langmuir 2010;26:2244–7.

[78] Akhavan O, Ghaderi E, Akhavan A. Size-dependent genotoxicity of graphene nanoplatelets in human stem cells. Biomaterials 2012;33:8017–25.

[79] Gurunathan S, Han JW, Eppakayala V, et al. Green synthesis of graphene and its cytotoxic effects in human breast cancer cells. Int J Nanomedicine 2013;8:1015–27.

[80] Zhi F, Dong H, Jia X, et al. Functionalized graphene oxide mediated adriamycin delivery and miR-21 gene silencing to overcome tumor multidrug resistance in vitro. PLoS One 2013;8:e60034.

[81] Sahoo NG, Bao H, Pan Y, et al. Functionalized carbon nanomaterials as nanocarriers for loading and delivery of a poorly water-soluble anticancer drug: a comparative study. Chem Commun (Camb) 2011;47:5255–8.

[82] Bao H, Pan Y, Ping Y, et al. Chitosan-functionalized graphene oxide as a nanocarrier for drug and gene delivery. Small 2011;7:1569–78.

[83] Wan M, Liu Z, Li S, et al. Silver nanocoaggregates on chitosan functionalized graphene oxide for high-performance surface-enhanced Raman scattering. Appl Spectrosc 2013;67:761–6.

[84] Sasidharan A, Panchakarla LS, Chandran P, et al. Differential nano-bio interactions and toxicity effects of pristine versus functionalized graphene. Nanoscale 2011;3:2461–4.

[85] Singh SK, Singh MK, Kulkarni PP, et al. Amine-modified graphene: thrombo-protective safer alternative to graphene oxide for biomedical applications. ACS Nano 2012;6:2731–40.

[86] Tan X, Feng L, Zhang J, et al. Functionalization of graphene oxide generates a unique interface for selective serum protein interactions. ACS Appl Mater Interfaces 2013;5:1370–7.

[87] Robinson JT, Tabakman SM, Liang Y, et al. Ultrasmall reduced graphene oxide with high near-infrared absorbance for photothermal therapy. J Am Chem Soc 2013;135:6825–31.

[88] Mullick Chowdhury S, Lalwani G, Zhang K, et al. Cell specific cytotoxicity and uptake of graphene nanoribbons. Biomaterials 2013;34:283–93.

[89] Liu JH, Yang ST, Wang H, et al. Effect of size and dose on the biodistribution of graphene oxide in mice. Nanomedicine (Lond) 2012;7:1801–12.

[90] Duch MC, Budinger GR, Liang YT, et al. Minimizing oxidation and stable nanoscale dispersion improves the biocompatibility of graphene in the lung. Nano Lett 2011;11:5201–7.

[91] Bianco A. Graphene: safe or toxic? The two faces of the medal. Angew Chem Int Ed Engl 2013;52:4986–97.

[92] Wang X, Podila R, Shannahana JH, et al. Intravenously delivered graphene nanosheets and multiwalled carbon nanotubes induce site-specific Th2 inflammatory responses via the IL-33/ST2 axis. Int J Nanomed 2013;8:1733–48.

[93] Yang K, Gong H, Shi X, et al. In vivo biodistribution and toxicology of functionalized nano-graphene oxide in mice after oral and intraperitoneal administration. Biomaterials 2013;34:2787–95.

[94] Yang K, Wan J, Zhang S, et al. In vivo pharmacokinetics, long-term biodistribution, and toxicology of PEGylated graphene in mice. ACS Nano 2011;5:516–22.

[95] Ma-Hock L, Strauss V, Treumann S, et al. Comparative inhalation toxicity of multi-wall carbon nanotubes, graphene, graphite nanoplatelets and low surface carbon black. Part Fibre Toxicol 2013;10:23.

[96] Wu Q, Yin L, Li X, et al. Contributions of altered permeability of intestinal barrier and defecation behavior to toxicity formation from graphene oxide in nematode Caenorhabditis elegans. Nanoscale 2013;5:9934–43.

[97] Zanni E, De Bellis G, Bracciale MP, et al. Graphite nanoplatelets and Caenorhabditis elegans: insights from an in vivo model. Nano Lett 2012;12:2740–4.

[98] Zheng W, Wang C, Li Z, et al. Unraveling stress-induced toxicity properties of graphene oxide and the underlying mechanism. Adv Mater 2012;24:5391–7.

[99] Chng EL, Punera M. The toxicity of graphene oxides: dependence on the oxidative methods used. Chemistry 2013;19:8227–35.