REVIEW ARTICLE

Toxicology data of graphene-family nanomaterials: an update

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Abstract

Due to its unique physical structure and chemical properties, graphene family nanomaterials (GFNs) and derived commodities have been widely used in commercial products, particularly biomedical applications, which has significantly increased the risk of human exposure. There exists significant evidence that GFNs are accumulated in a number of tissues and organs through different exposure pathways, and further cause toxicity manifested as lesions or functional impairment. Moreover, GFNs can be internalized by varing cell types and induce cytoskeletal disorders, organelle dysfunction, and interact directly with biological macromolecules such as DNA, mRNA and proteins, ultimately resulting in greater rates of cell apoptosis, necrosis and autophagic cell death. The toxicological effect of GFN is closely related to its lateral size, surface structure, functionalization, and propensity to adsorb proteins. Using major data published over the past four years, this review presents and summarizes state of current understanding of GFN toxicology and identifies current deficiencies and challenges. This review aims to help improve evaluation of the biocompatibility of GFNs and provides theoretical guidance for their safe application.

Keywords Graphene family nanomaterials · Charateristics · Toxicity · Mechanism of action · Physichemical property · Challenges

Introduction

Rapid development of nanotechnologies has led to an increasing number of occupational exposure assessments for engineered nanomaterials (ENM). However, our understanding of environmental exposure is less well understood.

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Introducing these new materials into the work environment and consumer products requires a safety assessment to better understand any potential influence on human health. Graphene materials, a new allotrope of carbon, are "nanomaterials" or "nanoparticles" with transverse dimensions ranging from a few nanometers to several hundred nanometers and thicknesses ranging from 1 to 10 nm (Han et al. 2016; Shen et al. 2012b). Due to their unique structure, large surface area and active physicochemical property, the large number of applications for graphene-based materials has attracted extensive attention from all walks of life since their discovery in 2004. In recent years, biomedical applications of graphene family nanomaterials (GFNs) have received increasing attention, particularly for use in cell imaging (Sun et al. 2008), drug and gene delivery (Hussien et al. 2018; Yao et al. 2017), tissue engineering (Langer and Vacanti 2016; Webber et al. 2015) and as biosensors (Muthukumaran et al. 2016).

As the use of nanomaterials has increased the risk of unintentional occupational or environmental exposure to GFNs has increased (Pelin et al. 2018). Due to the wide range of potential applications of GFNs in biomedicine, exposure can occur via a number of pathways including

intratracheal instillation, oral gavage, intraperitoneal injection, intravenous injection, and subcutaneous injection (Amrollahi-Sharifabadi et al. 2018; Erf et al. 2017; Mao et al. 2016; Park et al. 2017; Xu et al. 2016). In addition, GFNs can diffuse across biological barriers such as the blood-air, blood–brain, blood-testis and blood-placental barrier, accumulate in tissues and organs, and cause acute and chronic toxicity (Mendonca et al. 2016a; Mohamed et al. 2019; Roberts et al. 2016; Sawosz et al. 2014). Furthermore, a number of studies have suggested that exposure of aquatic organisms in the environment to GFNs can result in effects of toxicological concern (Hu et al. 2016; Manjunatha et al. 2018a; Meng et al. 2019; Zhou and Hu 2017). Because nanomaterials might bioaccumulate in aquatic organisms and can be consumed by humans, there is potential risk to human health from ingestion of contaminated foods. Currently, a number of studies have investigated their mechanisms of toxicity action. Graphene and its derivatives have large specific surface areas and special hydrophobic properties, and can enter cells through a variety of pathways including clathrin-mediated or caveolae-mediated endocytosis, pinocytosis and phagocytosis (Ou et al. 2016), and further induce cytotoxic effects such as cytoskeletal injury (Sasidharan et al. 2016; Tang et al. 2018), mitochondrial respiratory dysfunction (Jaworski et al. 2019; Park et al. 2015), lysosomal overload (Feng et al. 2018), oxidative stress (Gurunathan et al. 2019a, b; Srikanth et al. 2018), and inflammation (Fujita et al. 2018; Vranic et al. 2018). Moreover, evidence suggests that nanomaterials can directly interact with biological macromolecules such as DNA (Xu et al. 2018), proteins (Babadaei et al. 2018) and small RNA (Djurisic et al. 2015), and can induce apoptosis, necrosis and autophagy cell death (Fahmi et al. 2017; Yang et al. 2019). However, although preliminary progress has been made, there is a need to standardize and further investigate the toxicity of these novel materials as data from different laboratories, in vivo models, and in vitro experiments differ and are limited (Wu et al. 2018a; Yadav et al. 2017).

It is important to review and assess new toxicity information and identify potential hazards related to the use of new technologies (Shvedova et al. 2016). The current review mainly focuses on published toxicological information related to GFNs from 2016 to present. By comparing toxicity of GFNs and the underlying mechanisms of action in vivo and in vitro, this paper aims to provide an overview and suggestions for future research. Furthermore, it is hoped that with a greater understanding of the toxicity of GFNs, we can help improve the biosafety of GFNs and promote their wider applications.

Characteristics and applications of GFNs

Graphene and its derivatives mainly include monolayer graphene, few layers of graphene (FLG), graphene oxide (GO), reduced graphene oxide (rGO), and graphene quantum dots (GQD) (Fig. 1) (Chen et al. 2017; Dasmahapatra et al. 2019; Tu et al. 2018). FLG is a flaky stack of 2–10 graphene layers that was originally a by-product or precursor of monolayer graphene manufacturing. Sulfates, nitrates or other ions are embedded between the layers of crystalline graphite and then heated rapidly, resulting in increased internal pressure and substantial expansion of the graphite lamellar structure. The dry powder produced by hot stripping may result in occupational exposure (Sanchez et al. 2012). The majority of studies have focused on GO and rGO because they have better solubility and dispersion in water and physiologically relevant conditions when compared to other GFNs. GO is a highly oxidized chemically modified graphene whose carboxylic acid groups provide negative colloidal stability and charged surfaces. The surface of GO contains functional groups that provide π–π interactions, can adsorb drugs and can be used for molecular imaging (Park et al. 2009). RGO is the product of GO when subject to reducing conditions, such as heat and chemical treatment with hydrazine (N_2H_4), or other reductants. The RGO process is used to restore electrical conductivity and is characterized by reduced oxygen content and increased hydrophobicity (Bagri et al. 2010; Park et al. 2009). The novel zero-dimensional graphene nanomaterial, GQDs, has a number of advantages when compared to conventional organic photosensitizer (PS), such as improved biocompatibility, high water solubility and light stability, excellent optical properties and surface functionalization (Ko et al. 2017; Tabish et al. 2018). Therefore, GQDs have the potential to replace the commonly used QDS derived from metal sulfides (Zhao et al. 2020). In addition, ε-β bonds above and below the atomic plane of graphene provide excellent electrical and thermal conductivity when compared to conventional semiconductor quantum dots (Arvand and Hemmati 2017; Zhao et al. 2017).

Graphene and its derivatives are particularly attractive because they have unique properties of nanomaterial polymers including superior physical and chemical properties, electrical and thermal conductivity, and magneto-optical absorption (Lee and Lee 2017; Mahanta and Abramson 2012; Suk et al. 2010). For example, graphene can increase the mechanical strength and electrical conductivity of composites (Hu et al. 2013), showing prominent advantages for engineered neural tissue technologies (Nezakati et al. 2019; Homaeigohar et al. 2019). Similarly, gelatin-functionalized GO (GO-Gel) can be used
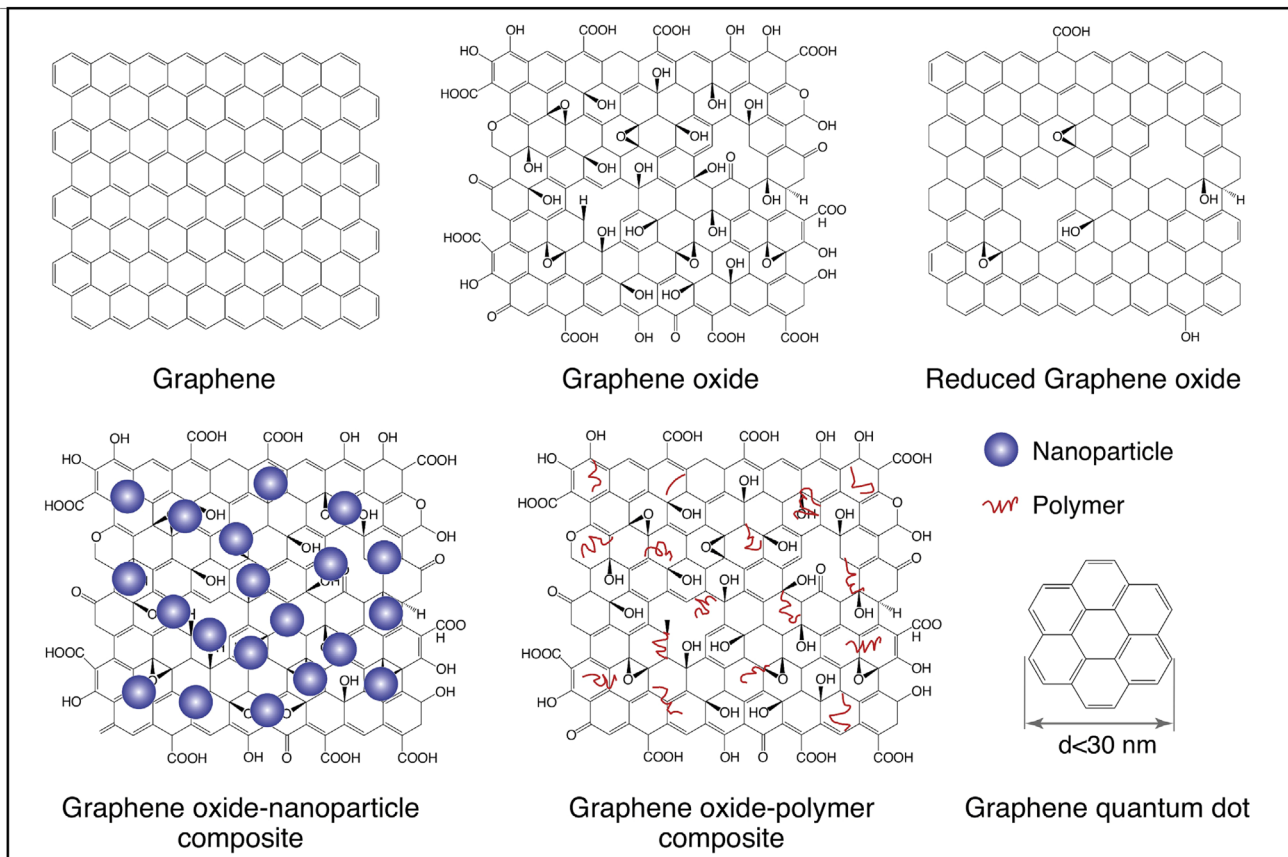
Fig. 1 Schematic illustration of different graphene-based nanomaterials. Reprinted with permission from Ref. (Zhao et al. 2017). Copyright Drug Discov Today

for biomimetic mineralization of hydroxyapatite, which promoted the adhesion, proliferation, and osteogenic differentiation of MC3T3-E1 cells (Liu et al. 2014). Currently, functionalization of GFN carriers has played an important role in drug and gene delivery due to improved biocompatibility, loading rate and release performance (Kundu et al. 2015; Wen et al. 2012; Zhao et al. 2015). Note that GFN-based nanocomposites revealed excellent protection against nuclease degradation of DNA and siRNA (Joo et al. 2016; Shen et al. 2012a) and better cellular permeability than viral vectors (Whitehead et al. 2009; Yin et al. 2017). Finally, graphene materials can also be used to target cancer cells for imaging by tagging them with folate (FA) (Huang et al. 2015; Maji et al. 2015), hyaluronic acid (Gui et al. 2018), proteins (Guo et al. 2016), and peptides (Su et al. 2015). Considering there are so much data demonstrating the GFN applications in biomedicine, their exposure risks to human health have increased significantly.

Toxicity in vivo

Exposure pathways and biodistribution

The fate of GFNs in exposed organisms is influenced by their physiochemical properties, as well as by the environment in which they come into contact with organisms (such as biocorona) (Bhattacharya et al. 2016; Docter et al. 2015). Moreover, the pathway of exposure (related to occupational, environmental exposure, and biomedical applications) can affect GFN metastasis, accumulation, degradation, and clearance.

Inhalation exposure

Inadvertent contact with graphene in occupational or environmental settings is increasing, and exposure is

dependent on the method of production and protective measures implemented (Heitbrink et al. 2015). Previously, some studies have reported that the maximum concentration of graphene in factories during manufacturing and processing is 2×10^6 particles/cm^3 (Lo et al. 2011). However, in another study, exposure to graphene particles was very limited with particle concentration was less than 40,000/cm^3 (Lee et al. 2016). Recent subchronic inhalation studies have suggested that the safe concentration for occupational exposure to graphene is 18 µg/m^3 (Lee et al. 2019). A large number of in vivo experiments have confirmed that inhaled GFNs are mainly deposited in the lungs, and can diffuse across the blood-gas barrier (Ema et al. 2016b; Krajnak et al. 2019; Park et al. 2017). More specifically, labeled ^{14}C FLG administered by intratracheal instillation accumulated in the lung tissues of mice, and radioactivity in the lungs, intestine, stomach and feces accounted for 85%, 3%, 1.5% and 4.6% of the total dose 1 day after exposure, respectively. In addition, concentration of FLG in the lungs, intestine and stomach decreased over time, while excreted FLG gradually increased in feces. At 28 days after exposure, 46.2% of FLG was excreted via feces (Li et al. 2013).

Intravenous injection

After intravenous injection, GO is distributed throughout the body via blood. How to detect the distribution of large molecular weight nanomaterials in vivo is one of the research focuses. Common nanomaterials imaging methods can be divided into two categories. The first is labeling methods, such as radioisotope (Georgin et al. 2009) and fluorescent labeling (Huang et al. 2013). This class of methods is time consuming and can cause tag separation over time (Liu et al. 2008). Distribution in the body can also be monitored using the inherent physical/chemical properties of nanomaterials such as via Raman imaging (Syama et al. 2017). Traditional observation techniques may be limited by slow imaging speeds, weak luminescence signals and strong background interference. Recent studies have reported a novel unlabeled technique, matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging (MSI), which has a high imaging speed, high sensitivity, and suborgan quantitative ability (Chen et al. 2015; Ellis et al. 2014). The ability to use suborgan quantitative information augments insights into bio-nano interactions and pharmacokinetics of chemicals.

Intrapritoneal injection

Wistar rats injected intraperitoneally with GO (doses of 50, 150 or 500 mg/kg) demonstrated a dose-dependent distribution among the liver, kidney, spleen, lung, intestine, and brain (Amrollahi-Sharifabadi et al. 2018). Another report provided a more detailed analysis of the biodistribution of intraperitoneal injected ^{125}I-GO-PEG. GO-PEG mainly concentrated in the liver, spleen and bone 1 h after intraperitoneal injection. Note that high levels of radioactivity was detected in urine and feces, suggesting that the removal of GO-PEG may be through kidney and fecal excretion (Yang et al. 2011). However, we believe the decrease of GO-PEG in organs might be due to isolation of markers over time. In another mice study, intraperitoneally administered PrGO was mainly detected in the brain, liver, spleen, kidney and bone marrow monitored by Raman spectroscopy. Urinalysis showed a weak PrGO signal, indicating a low rate of excretion of PrGO by the kidneys, which is consistent with the current understanding that PrGO is excreted primarily through the biliary tract and can cause liver damage (Syama et al. 2017).

Oral gavage

To study the effects of gastrointestinal exposure, Mao et al. (2015) gave male ICR mice FLG via oral gavage (0.1 mg/mL) for 3 days. After 12 h of exposure, the amount of radiation in the stomach, intestine and feces accounted for 3%, 6% and 85% of the total dose, respectively. After 48 h of exposure, most of the material (> 98%) was excreted through feces. No radioactivity was detected outside the blood and digestive tract, suggesting that FLG did not diffuse into the blood at detectable concentrations through the gastrointestinal tract. In contrast, after the first day of exposure, oral administration of ^{125}I labeled rGO was rapidly absorbed in the gastrointestinal tract, metabolized by the kidneys and then excreted via urine (Zhang et al. 2015). In addition, the influence of changes in intestinal flora on host health has received extensive attention (Chen et al. 2018; Nakanishi et al. 2015; Ormerod et al. 2016). When compared with a control group, the relative abundance of Firmicutes in mice exposed to FLG decreased by $10 \pm 1.2\%$, while the relative abundance of bacteroides increased (Mao et al. 2016). However it is important to note that ingested graphene may be coated with surfactants or biomolecules in the lungs or digestive system. Therefore, further studies are needed to elucidate the effects of long-term exposure to graphene in the digestive system on gut microbes and how this relates to organism health.

Dermal exposure

Skin is one of the main barriers between the body and the environment. Previous study reported that subcutaneously injected rGO and GO induced significant mononuclear cell infiltration near the injection site. Furthermore, the more hydrated GO had weaker interactions with macrophages, suggesting that

the immune response was related to functional groups on the surface of the material (Sydlik et al. 2015). Recent studies have investigated cellular/tissue damage locally and throughout the body by injecting GFNs into the dermis of chicken growth feathers (GF) (Erf et al. 2017). Compared with the traditional subcutaneous injection, the use of skin derivative GF as a skin test site has the advantages of being easier to operate and less invasive (Erf and Ramachandran 2016). Seven days after a single injection, continuous infiltration of white blood cells occurred at the injection site, but no significant abnormality in white blood cell spectra of peripheral blood circulation was observed (Duch et al. 2011; Wang et al. 2011b). These results highlighted the causes of localized immune responses following GFN dermal exposure.

Eye irritation

Studies on the health risks of GO eye exposure are also limited. Unilateral eye drop stimulation studies using Sprague–Dawley rats administered GO (12.5, 25, 50 or 100 g/mL) demonstrated temporal and dose-dependent ocular injury including corneal epithelial necrosis shedding and corneal stroma exposure (Wu et al. 2016). However, other studies observed that exposure to graphene and GO did not result in significant ocular toxicity (Lin et al. 2015; Yan et al. 2012). In addition to potential exposure via liquid splashes in occupational settings, exposure via gaseous vapors are possible. Although some inhalation studies have reported toxic effects following exposure to gaseous GFNs, ocular toxicity has not been reported (Han et al. 2015; Kim et al. 2016; Park et al. 2015). More studies are needed in the future to explain the diversities among different studies and labs.

Overall, the route of GFN administration affected the pharmacokinetics in the body. Intravenous injection of PrGO resulted in direct transfer from blood to the liver and spleen, while intraperitoneal injection of PrGO resulted in accumulation in the liver (Syama et al. 2017). Oral administration of GFNs to mice resulted in minimal absorption and distribution among organs via the digestive tract, but nanomaterials partitioned to the liver and spleen when injected or following intratracheal instillation (Mao et al. 2016; Yang et al. 2013). Though the inhalation method provides the most realistic simulation to real life exposure, instillation is more effective method, and GFNs was found that causing longer inflammation period using instillation than inhalation (Lee et al. 2017; Li et al. 2013; Schinwald et al. 2012, 2014).

Systemic toxic behavior

Lung toxicity

Numerous studies have reported the lung toxic effects of inhaled GFNs, including alveolus-capillary barrier damage (Hu et al. 2015), local inflammatory cell infiltration and release of pro-inflammatory cytokines (Duch et al. 2011), mitochondrial damage and down-regulation of reactive oxygen species (Park et al. 2015, 2017). Toxicity of graphene nanomaterials depends on their different physiochemical properties (Huang et al. 2012; Zhang et al. 2013, 2016). For example, immune and inflammatory responses were found to be most significantly increased following exposure to the larger graphite nanoplates Gr20 (12 μm) and Gr5 (5 μm) in pharyngeal aspirated mice (Roberts et al. 2016). Surface structure is another influencing factor. The toxicity of inhaled GFNs is FLG < GNP (graphite nanoplates) < rGO < GO, where the inflammatory effect of positively charged GNP was slightly greater than that of the negatively charged GNP (Bengtson et al. 2017; Ema et al. 2017; Lee et al. 2017; Roberts et al. 2016). Other involving factors include oxidation state and surface carbon free radical density (Hu et al. 2010; Ji et al. 2010; Li et al. 2018b).

Neurotoxicity

At present, a large body of evidence demonstrated that GFNs could cross the blood–brain barrier (BBB) and resulted in central nervous system (CNS) toxicity (Mendonca et al. 2016b; Rauti et al. 2016). For example, the leakage of Evans blue dye from capillaries in the hippocampus was not detectable on the 7th day after injection into the tail vein of rats, suggesting that toxicity of rGO was reversible. However, density of the BBB differs among the same blood region of the hippocampus further complicating interpretations (Mendonça et al. 2015b; Raleigh et al. 2010). Functionalized graphene family materials are also reported to be capable of penetrating the BBB (Georgakilas et al. 2016; Reina et al. 2017). Intravenous injected rGO-PEG were detected in the hippocampus and thalamus of rats, and the BBB was considered to be temporarily open following continuous decline of the junction proteins, laminin and Cx43 (Mendonca et al. 2015a). The authors further indicated that the nervous system toxicity induced by rGO-PEG was closely related to high levels of cellular reactive oxygen species (ROS) (Mendonca et al. 2016a). More importantly, lack of Cx43 expression or Cx43 channel blockage could aggravate ROS-induced astrocyte death (Le et al. 2014). Notably, the aggregation of GFNs in the

CNS increased with time, while this cumulative effect was infrequent in other organs (Baldrighi et al. 2016). The slow accumulation and long-term persistence of GFNs in CNS is an advantage as a drug delivery system, but also raises concerns about their chronic toxicity.

Reproductive toxicity

The blood-testes and blood-epididymis barriers are among the densest blood-tissue barriers in mammals (Mital et al. 2011). The potential effect of graphene exposure on reproduction varied greatly among animal models and materials. Liang et al. (2015) found that GO could not cross the blood-testes barrier and did not result in any reproductive toxicity, which results were consistent with GQD exposure on the reproductive ability of male mice and their offspring (Zhang et al. 2019). In contrast, accumulation of GO in the testicles has been reported to result in a significant decrease in sperm motility in the epididymis, sperm DNA damage and an increase in ROS production (Akhavan et al. 2015). Although exposure to GO resulted in structural abnormalities in the testis, but a gradual recovery was observed within 30 days and fertility of the rats was not significantly affected (Nirmal et al. 2017). Note that rGO did not alter concentrations of estrogen in the serum of non-pregnant female mice, while mice in the late stages of pregnancy exposed to rGO resulted in loss of fetus and mother (Xu et al. 2015).

Embryotoxicity of graphene and its derivatives has emerged as an important consideration. It has been suggested that rGO is virtually absent in the placenta and fetus during the third trimester of pregnancy after intravenous administration (Xu et al. 2015; Yang et al. 2012). However, other reports have observed transplacental metastasis during the third trimester of pregnancy (Huang et al. 2014; Qi et al. 2014) Moreover, developmental toxicity of GFNs was confirmed by their ability to cross the placental barrier and strongly impact the development of embryos (Ema et al. 2016a; Teng et al. 2020; Warheit et al. 2015; Zhou et al. 2015). Exposure to GO via oral gavage during lactation led to stunted growth in mouse offspring (Fu et al. 2015). However, results were difficult to interpret as mother mice drank less water in the high GO dose group which might have led to lesser production of milk, thus retarding offspring growth (Fu et al. 2015; Gao and Oba 2014). In zebrafish, similar to mammalian models, reproductive toxicity of graphenes with differing physicochemical properties significantly differed (Gollavelli and Ling 2012; Manjunatha et al. 2018b).

Other visceral toxicities

Nanomaterials such as GO may cause acute inflammatory responses and chronic injuries by interfering with normal physiological functioning of vital organs (Li et al. 2013;

Wen et al. 2015). For example, in mice injected with intravenous or intraperitoneal PrGO, livers presented with granular cytoplasms, vacuolization, and denaturation, followed by mononuclear infiltration in the kidney and extramedullary hematopoiesis in the spleen (Syama et al. 2017). Oral administered GO nanoparticles produced dose-dependent liver and brain damage, with histological changes including increased apoptosis, necrosis, inflammation, and cellular degeneration (Mohamed et al. 2019). Differences in surface structure impact observed toxic effects in affected organs. Pristine GO exposure resulted in increased H_2O_2 concentration in the heart, whereas no significant change were detected when exposed to unoxidized graphite nanocrystals (Krajnak et al. 2019). Toxicity differences may result from the hydrophilic properties of GFN for materials possessing oxygen-containing group are more easily absorbed by cells (Chatterjee et al. 2014). The formation of protein corona can also affect internal organs toxicity. Compared to poly (acrylic acid)-functionalized GO (GO-PAA) and GO-PEG, intravenous injected GO-PAM with higher level of IgG (50–70%) demonstrated the highest liver and lung toxicity.

Blood toxicity

Most administration routes of nanomaterials can lead to increased concentrations in the blood. Mice oropharyngeal aspiration after exposure to GO and rGO, the arteries for vasoconstriction induced by epinephrine is more sensitive. In light of these results, the authors suggested that the observed cardiovascular and renal effects might be due to pulmonary inflammation and production of ROS following exposure to graphene (Krajnak et al. 2019). Furthermore, fragmented muscular layers of the small capillary wall (hyalinization) and fragmented larger blood vessels (microthrombi formation and endothelial swelling) were also observed following GO intraperitoneal injection (El-Yamany et al. 2017). Note that submicron sized GO had the greatest hemolytic response, with significant platelet aggregation, while larger material had a lesser hemolytic response likely because the material tends to aggregate (Li et al. 2014). In addition, oxidized graphene has more significant acute effects on the vascular and renal systems when compared to non-oxidized forms (Krajnak et al. 2019).

Immunotoxicity

Exposure to GFNs might disrupt the immune system and result in progression of certain diseases (Luo et al. 2017; Rodrigues et al. 2018; Shurin et al. 2014). Intravenous injected graphene nanosheets (GNS) activated Th2 type immune responses via interleukin-33 (IL-33) and ST2 receptors in the lung (Wang et al. 2013a). GNS exposure can also induce Th1-shifted immune responses and lung cytoskeleton

damage (Park et al. 2017). In addition, evidence suggested that macrophages had a size dependent mechanism of GFN uptake (Rodrigues et al. 2018; Wang et al. 2013b).

Genotoxicity

The genotoxicity of GO is characterized by various types of structural chromosomal aberrations, which are both dose and time dependent (Bengtson et al. 2017; Mohamed et al. 2019). Recent studies revealed that intraperitoneal injected GO resulted in mitotic abnormalities, DNA damage (strand breakage), chromosome deletion and chromosome fracture in lung tissues (El-Yamany et al. 2017). The observed genotoxic effects were largely due to nanomaterial-triggered oxidative stress and reduced mitochondrial membrane potential (Manke et al. 2013; Patlolla et al. 2016; Wang et al. 2011a) and mechanical damage (GO may be inserted between base pairs of DNA) (Ren et al. 2010; Stueckle et al. 2016). However, in vivo studies on genotoxicity of graphene are still limited and require further investigation to elucidate their genotoxic effects via RNA sequencing (Table 1).

In vitro toxicity

The response of cells to GFN exposure are dependent on biological interactions with the plasma membrane, followed by possible cellular uptake and potential interactions with subcellular structures (Zhang et al. 2016). Toxicity data of graphene-based nanomaterials in mammalian cells are summarized in Table 2.

Interactions with plasma membrane

Numerous studies have observed that GO co-cultured with different types of cells stick to the cell surface and enveloped by the plasma membrane (Feng et al. 2018; Kalman et al. 2019; Tang et al. 2018). Interactions of GO with the cellular lipid bilayer is largely due to the amphiphilic nature of the material, which possesses a hydrophobic planar structure with hydrophilic edges (Kim et al. 2010). When compared to GO, rGO is more hydrophobic in nature due to a decreased number of hydrophilic groups which may be more easily internalized by phagocytic uptake (Li et al. 2018b). After adhesion to cell membranes, GFNs could diffuse into the lipid bilayer or be internalized in the cell via uptake mechanisms, thus causing a physical or biological damage to the cell membrane. Multilayer graphene nanoflakes were found to be capable of extruding phospholipids from the bilayer of cells, resulting in lipid consumption and formation of permeable pores and final cell death (Duan et al. 2017). The interaction of GFN with the plasma membrane differ significantly with GFN type and treatment conditions.

Pristine GO is capable of triggering lipid peroxidation and membrane integrity damage without being internalized by cells (Li et al. 2018b). In comparison, when dispersed in cell medium, the amount of free radicals (especially carbon free radicals) on the surface of GO increases significantly, which could interact with cell membranes, leading to adverse effects on cell viability (Vranic et al. 2018). Additionally, GFNs have been observed to damage the integrity of cell membrane structure through regulation of expression levels of membrane- and cytoskeleton-associated genes (such as Actg2, Myosin, Tubb2a, and Nebuli) (Xu et al. 2016; Lammel et al. 2013; Gurunathan et al. 2013b).

Uptake and intracellular distribution

Biological interactions between GFNs and cytomembranes are likely to result in cellular uptake via clathrin-mediated or caveolae-mediated endocytosis, pinocytosis and phagocytosis (Ou et al. 2016; Seo et al. 2017). Intracellular uptake of GFNs is largely influenced by their physiochemical properties such as: particle size, surface charge, shape as well as by cell type (i.e. fibroblast, macrophage and neuronal cells) (Adjei et al. 2014; Bramini et al. 2016; Sydlik et al. 2015). For example, lateral GO flake size effects cellular interactions of larger sized GO as their uptake is hindered by their size (Ma et al. 2015). Moreover, smaller sized low-reduced GO particles (LRGO) may be more easily internalized by the myocardial cell line H9c2 and distributed near the nucleus, suggesting an endocytic process of internalization (Contreras-Torres et al. 2017). Surface modification of functional groups modulates cellular uptake by changing surface hydrophobicity/hydrophilicity and charge of GFNs (Xu et al. 2016). A series of studies by Xu et al. found that AG-QDs can enter rat alveolar macrophages (NR8383) via energy-dependent endocytosis, phagocytosis and caveolae-mediated endocytosis as regulated by the nuclear pore complex (NPC) genes, karyopherin $\beta2$ (Kap$\beta2$) and nucleoporin 98 (Nup98). Additionally, the AG-QDs are localized in the cytoplasm and nucleus, and resulted in nuclear membrane shrinkage and deformation of nuclear morphology (Xu et al. 2018, 2019). In comparison, exfoliated graphene (EGr) partitioned to the cytoplasm and nucleus following uptake by NR8383 cells (Fujita et al. 2018). Differences in shape of GFNs appear to result in distinct patterns of localization within cells. GO nanosheets may be present within membrane encompassed vesicles and in their free form in the cytoplasm, while shorter carbon nanofibers were localized in vesicles (Kalman et al. 2019). Interestingly, TEM images further demonstrate deformations of intracellular materials, indicating that the flexibility of GO sheets impact cellular uptake pathways. Currently, evidence also suggested partitioning of GFNs to the cytoplasm of cells, including inside endo- and lysosomes next to the Golgi apparatus and in the

Table 1 Toxicology data of graphene-based materials in mammal animals

Graphene family nanomaterier	Physiochemial properties and functionalization	Animals	Dose and time incubation	Effects	References
Graphene oxide (GO)	Size: 1162 nm, thickness: 62.5 ± 51.42 nm	Male swiss webster mice	10, 20, 40 mg/kg, oral gavage, 5 or 10 consecutive days	Induced chromosomal, DNA damage, and tissue lesions in the brain and liver tissues	Mohamed et al. (2019)
Carbon black(CB); Graphene (Gr); GO; Reduced graphene oxide (rGO)	CB: size of 15–20 nm, Gr1: size of 1–2 μm, thickness: 1–2 nm, Gr5: size of 5 μm, thickness 7 nm, Gr20: size of 20 μm, thickness: 7 nm, GO: size of 5 μm, thickness: 7 nm, rGO: not reported	C57BL/6J mice	40 μg, oropharyngeal aspiration, 15 seconds	Alterations in ROS and gene expression, leading to cardiovascular dysfunction	Krajnak et al. (2019)
GO	Size: 5–10 μm, thickness: 0.8–2 nm	Male Wistar rats	50, 150, 500 mg/kg, intraperitoneally injected, every 48 hours during 1 week	Small foci of neuronal degeneration and necrosis in the brain, granuloma reactions in the internal organs, giant cell formation and GO accumulation in the capsule area	Amrollahi- Sharifabadi et al. (2018)
Few-layer graphene (FLG)	Size: 60–590 nm thickness: 0.97–3.94 nm	Male ICR mice	10 μg, oral gavage; 5 μg, intratracheally instilled, single administration	Intratracheally instilled graphene was mainly retained in the lung, graphene with oral gavage did not detectable absorption through the gastrointestinal tract	Mao et al. (2016)
GO	Size: 50–500 nm, thickness: 2–15 nm	Light-brown Leghorn	10 μL, intradermal injection, GFs* were collected before-(0) and at 0.25, 1, 2, 3, 4, 5, and 7 days	Local cellular reactions and inflammatory reactions were observed, the distribution of white blood cells in peripheral blood showed no abnormal	Erf et al. (2017)
GO	Size: 120 nm thickness: <1.2 nm	Albino rabbits	50, 100 μg/mL, eye irritation, 5 consecutive days	Induced reversible damage to the eye via oxidative stress, alleviated by the antioxidant GSH	Wu et al. (2016)
Graphene	Size: <2μm thickness: 0.97–3.94 nm	Male Sprague–Dawley rats	0.12, 0.47 and 1.88 mg/m³, nose inhalation, 6 hours /day, 5 days/week for 4 weeks	Graphene was mostly ingested by macrophages, no distinct lung pathology was observed	Kim et al. (2016)
PEGylated reduced graphene oxide (PrGO)	Size: 1 μm, thickness: 4–9 nm	Albino mice	10 mg/kg,injected intravenously and intraperitoneally, single administration, 3 consecutive days	Accumulated in major organs including the liver, spleen and kidney, causing hepatotoxicity and immune responses	Syama et al. (2017)

Table 1 (continued)

Graphene family nanomaterier	Physiochemial properties and functionalization	Animals	Dose and time incubation	Effects	References
Graphene	Gr1: Size <2 μm Gr5: Size 5 μm Gr20: Size 20 μm thickness: 8–25 nm	Male C57BL/6 J mice	4 or 40 μg, pharyngeal aspiration two full breaths (not longer than 15 seconds)	Graphene with larger lateral dimensions and increased surface reactivity caused injury and inflammation in the lung	Roberts et al. (2016)
GO; RGO; Hydrated GO (hGO)	Size: 100–150 nm, thickness: 0.8–1.6 nm	C57Bl / 6 mice	2 mg/kg, pulmonary aspiration, 40 hours	RGO induced the lowest level of lung inflammation than other graphene materials	Li et al. (2018b)
GO	Size: 5–10 μm, thickness: 0.8–2 nm	Male Wistar rats	0.4 mg/kg, 2.0 mg/kg, 10.0 mg/kg, intraperitoneally injected, 15 or 30 consecutive days	10.0 mg/kg of NGO resulted in damage to testicular tissue	Nirmal et al. (2017)
GO GO-NH2 GO-PAM GO-PAA GO-PEG	Size: 100–500 nm PAM thickness: 2.9 nm PAA thickness: 2.6 nm PEG thickness: 4.1 nm GO thickness: 1.1nm	Male BALB/c mice	0.05, 0.25, 0.5, 1, 2, 5, 10 and 20 mg/kg, intravenous injection, single administration	PAA-modification induced the greatest biocompatibility; PAM-modification induced the most significant toxic effects	Xu et al. (2016)
GO	Size: 0.5–5 μm, thickness: 1 nm	Male Sprague–Dawley rats	0.76 ± 0.16 mg/m^3, 2.60 ± 0.19 mg/m^3, 9.78 ± 0.29 mg/m^3, nose inhalation, 6 hours/day, 5 consecutive days	Kidney, liver, and lung functions showed no significant toxicological significance	Dziewięcka et al. (2017)

*Chicken growth feathers

Table 2 Toxicology data of graphene-based materials in cell models

Graphene family nanomaterias	Size and functionalization	Cell lines	Dose and time incubation	Toxic effects	References
FLG	Lateral size:160 ± 48.5 nm, thickness: 0.8 ± 0.42 nm	Human primary umbilical vein endothelial cells (HUVEC)	2.5, 5, 10 µg/mL for 24 h	Induced organized oxidative stress paradigm, actin cytoskeleton contraction, DNA damage and cell death by apoptosis/necrosis	Sasidharan et al. (2016)
Pristine graphene platelets (GPs)	Lateral size: 420 nm to 1.6 µm, agglomerates: > 4 µm	U87 glioma cells and non-cancer HS-5 cell lines	20, 50, 100, 200 ug/mL for 1, 4, 12, and 24 h	Induced dose-dependent cytotoxicity via ROS over-production and depletion of the mitochondrial membrane potential	Jaworski et al. (2019)
GO	Lateral size: 500–800 nm, thickness: 1 nm	Rat pheochromocytoma-derived PC12 cell line	5, 10, 20, 30, 40, 50, and 60 µg/mL for 6, 12, 24 h	Induced impairment of autophagic flux, lysosomal dysfunction and p62-dependent cell apoptosis	Feng et al. (2018)
GO	Lateral size: 50 nm in water, 90 nm in medium	Human embryonic kidney (HEK293)	10, 20, 30, 40, 50 µg/mL for 24 h	Induced alterations of gene and transcription factor associated with apoptosis biological pathways, oxidative stress and lipid peroxidation	Gurunathan et al. (2019a)
GO	Lateral size: 100 and 20 nm	Leydig cells (TM3) and Sertoli cells (TM4)	10, 20, 40, 60, 80, and 100 µg/mL for 24 h	Induced ROS pruduction, DNA damage and apoptosis through phosphorylation levels of EGFR/AKT;	Gurunathan et al. (2019b)
Graphene quantum dots (GQDs)	Average size: 4.1 nm, thickness: 0.72 nm in DI water, average size: 9.40–11.8 nm, thickness: 4.30–10.2 nm in medium, aminated graphene QDs (AG-GQDs)	Rat alveolar acrophages (NR8383)	10, 25, 50, 100 and 200 µg/mL for 24, 48, 72 and 96 h	Induced DNA cleavage, ROS elevation, caspase gene up-regulation and early apoptosis	Xu et al. (2018)
GO nanosheets	Lateral size: 20 nm	Human lymphocyte cell	1, 10, 20, 50, and 100 µg/mL for 24 h	Induced cell circle arrest and apoptosis, human hemoglobin (Hb) structure alteratiomn	Babadaei et al. (2018)
RGO	Thickness of 1.5nm	Lung cancer cells A549 and SKMES-1	5, 50, 250, 500 and 1000 µg/ml for 24 h	Induced late apoptosis and necrosis through disintegrating the cellular membranes in a dose dependent manner	Tabish et al. (2018)
GO	Hydrodynamic sizes: 256–673 nm	Topminnow fish hepatoma cell line (PLHC-1), carp leukocyte cell line (CLC)	3.18, 12.5, 50 and 200 µg/ml for 24 and 72 h	Induced alteration of metabolic activity, impared plasma membrane integrity and lysosome functionality	Kalman et al. (2019)

Table 2 (continued)

Graphene family nanomaterials	Size and functionalization	Cell lines	Dose and time incubation	Toxic effects	References
1 and 4 layer GO nanoflake	Length: 1–25 µm, thickness: 1.0 nm of 1 layer and 3.0 nm of 4 layer, PEGylated	Human caucasian breast adeno-carcinoma cells (MCF-7)	3.125, 6.25, 12.5, 25, 50, and 100 µg/mL for 48 h	Induced stronger inhibition of cell proliferation and higher ROS generation by 1 layer GO-PEG	Peruzynska et al. (2017)
GO	Length: 3±1.2 µm, height: 25±15 nm	NIH3T3 fibroblasts	10, 30, 50 µg/mL for 2 and 24 h	Induced production of ROS, disruption of cytoskeleton, decreased cell's stiffness (Young's modulus)	Pastrana et al. (2019)
GO	Lateral size: 1–20 µm, thickness: 0.7–1.2 nm	Rat cardiomyoblast cell line H9c2	20, 40, 60, 80, and 100 µg/mL for 24 h	Induced mitochondrial disturbances, generation of reactive species and interactions with DNA	Arbo et al. (2019)
GQDs	Lateral size: 4–6 nm, thickness: 1–3 nm, hydrodynamic size: 14.1 ± 2.3 nm, hydroxyl-modified GQDs (OH-GQDs)	Human esophageal epithelial cell line HET-1A	25, 50, 100 and 200 µg/ml for 24 and 48 h	Blocked in G0/G1 cell cycle phase, disrupted microtubule structure and inhibited microtubule regrowth	Li et al. (2018a)
Graphene nanoplatelets	Lateral size: 1–2 um, thickness: < 3 layers, depyrogenated graphene	Mouse macrophage J774 cells	1 and 20 µg/ ml for 1, 3 and 48 h	Induced changes of gene expression for TLR signaling, NOD-like receptor signaling, and downstream signal transduction molecules	Lahiani et al. (2017)
Graphite powder, nanographene	Five different diameters, lateral size:50 nm–500µm	Human epithelial Caco-2 cell line	50, 100, 150, 200, 250, 300, 350 µg/mL for 5 days	Induced size-dependent cytotoxicity and ATP depletion	Saha et al. (2016)
GQDs	Three different diameters, 3.5–5 nm, cGQDs (COOH-GQDs), hGQDs (OH-GQDs), aGQDs (NH_2-GQDs)	Lung carcinoma A549 cells	10, 20, 50, 100 and 200 µg/mL for 24 h	Induced autophagy through activated p-p38MAPK, inhibited p-ERK1/2 (aGQDs and hGQDs) and activated p-JNK(hGQDs)	Xie et al. (2019)

perinuclear region (Kersting et al. 2019; Peruzynska et al. 2017).

Mechanisms of intracellular toxicity

Destruction of the cytoskeleton

Cellular morphology, motility and ability to adhere are closely associated with cytoskeletal alterations (Sasidharan and Monteiro-Riviere 2015; Tay et al. 2014). It has been reported that following 24 h-treatment of FLG resulted in significant alterations in cytoskeletal architecture and actin fiber stress in HUVECs cells (Sasidharan et al. 2016). Effects on cytoskeleton structure and integrity following to GFNs are highly sensitive to length of exposure and dose. For instance, short-term exposure of GO over 2–4 h resulted in inflated and riddled K_7M_2 cells, large numbers of vacuoles in the cytoplasm and cells became disordered and loosely adhered to their substrates (Tang et al. 2018). Following prolonged exposure of 24 h, cells experienced shrinkage and more irregular appearance, no longer adhered to the culture plate, even became floated in the medium(Gurunathan et al. 2019b; Srikanth et al. 2018). Similarly, as dose of GO increased from 5, 10 to 20 μg/mL, less cells were present and they had lesser cellular networking. At concentrations of 30 μg/mL or higher, increasing amounts of cell showed shrinkage until few viable cells were present (Gies and Zou 2017). When compared to nanometer sized GO, micrometer-sized GO induced more actin cytoskeleton remodeling in areas of the cell membrane in contact with the material, ultimately resulting in increased membrane blebbing and apoptotic cell death (Vranic et al. 2018). Functional graphene materials demonstrated similar damages on the cytoskeleton system. Treatment of cells with OH-GQD at 25 μg/mL resulted in disordering of the microtubule system. When the working dose increased to 100 μg/mL microtubule structure completely disintegrated (Hydroxylated-Graphene Quantum Dots Induce DNA Damage and Disrupt Microtubule Structure in Human Esophageal Epithelial Cells). These observations suggest that OH-GQD may participate in the dynamic regulation of microtubules. Notably, monitoring mechanical properties of cells has helped elucidate potential hazards to the filamentous actin cytoskeleton. Following treatment of GO flakes in NIH3T3 fibroblasts with 50 μg/mL, atomic force microscopy (AFM) demonstrated that cell stiffness (Young's modulus) significantly declined in a time-dependent manner, and was closely related to a high rate of ROS formation and disruption of the F-actin cytoskeleton (Pastrana et al. 2019).

Damage of cellular components

Substantial evidence have suggested that GFN exposure resulted in damage to mitochondria, especially on their aerobic respiration functions in cancer and non-cancer cell lines (Jaworski et al. 2019; Park et al. 2015). For instance, GO could result in depolarization of mitochondria in cardiac muscle cells, resulting in a reversed proton flux through the respiratory chain and excessive production of ROS (Arbo et al. 2019). Similarly, exposure of MHS cells to GO disturbed normal mitochondrial respiration by increasing the activity of the electron transport complexes I/III and the supply of electrons to site I/II, resulting in increased ROS formation (Duch et al. 2011). Lysosomes are important digestive organoids in cells and help maintain safe levels of foreign bodies. At present, evidence indicates that GO may enter cells by endocytosis, and accumulate in lysosomes in large quantities and further cause lysosome membrane destabilization and degradation disorder (Kalman et al. 2019; Wan et al. 2013). Lysosome-based degradation is closely related with cell autophagy and is thought to be an adaptive response for cell survival. Our previous work confirmed that although GO nanosheets can activate autophagosome formation through the conversion of LC3-I to LC3-II, the degradation of autophagic cargo p62 protein was inhibited due to lysosomal alkalization, ultimately leading to cell death (Feng et al. 2018). The ubiquitin–proteasome system is another pathway involved in intracelluar degradation. GO could adsorb 20S proteasome due to its hydrophobicity and caused dose-dependent inhibition of proteolytic activity of proteasomes, leading to adverse effects on cellular circle and survival (Ma et al. 2018). Identification of GO-triggered functional disturbance of the 20S proteasome provides a potential novel cancer therapy for treatment of cancers with abnormal proteasome activities. Additionally, gene ontology analysis has demonstrated that GO-PEG-NH$_2$ significantly alters ribonucleoprotein complex related gene expression of the ribosome and its subunits when compared with other cellular components (Wu et al. 2018b). There results are consistent with other research in which GO treatment led to dysregulation of proteins associated with ribosomal subunit (Yang et al. 2019). Unfortunately, limited works have focused on the effect of GFNs on other organelles such as the endoplasmic reticulum and the Golgi body, which may serve as a valuable research direction in the future.

Oxidative stress

Oxidative stress is a major mechanism of nanomaterial-induced toxicity in a variety of cell types, including bacterial and mammalian cells (Akhavan and Ghaderi 2010; Gurunathan et al. 2012, 2015b). Currently a large body of evidence exists to support dysregulation of cellular redox

balance following exposure to GFNs. For example, Sasidharan et al. (2016) found that exposure of human primary endothelial cells to FLG significantly increased the concentration of mitochondrial ROS, leading to oxidative degradation of lipids in the cell membrane and dose-dependent GSH oxidation. Similarly, naked graphitic platelets catalyzed electron transfer and production of superoxide ions and decreased cell viability, and was found to be largely size dependent (Zerbi et al. 2017). Functionalized graphene-based materials, such as PEG-GO-NH$_2$ could also downregulate NDUFA7 and NDUFB9, leading to dysfunction of mitochondrial complex I and accumulation of mitochondrial ROS (Wu et al. 2018b). Oxidative stress related cytotoxicity includes cell membrane damage, initiation of lipid peroxidation, covalent chemical modifications of nucleic acids, DNA-strand breaks, activation of transcription factors and modulation of inflammation (Gurunathan et al. 2019a, b; Srikanth et al. 2018). On the other hand, cells have developed a number of defenses mechanisms to maintain oxidative balances. It has been observed that ROS formation in MG-63 cells was suppressed by activation of the antioxidative factor, nuclear factor-E2-related factor-2(Nrf2), which translocated from the cytoplasm to the nucleus following GO exposure (Tang et al. 2018).

Inflammatory response

Broad activation of inflammatory responses and production of cytokines have been observed in a variety of cells exposed to GFNs. For example, expression of pro-inflammatory cytokines, such as MIP-1α, IL-1β, IL-18 and TNF-α significantly increased following exposure of rat alveolar macrophage cells NR8383 to exfoliated graphene (EGr) (Fujita et al. 2018). Similarly, exposure of human bronchial epithelial cell line BEAS-2B to large-sized GO substantially upregulated gene expression of IL-6 and IL-8 (Vranic et al. 2018). Pristine graphene (Zhou et al. 2012) and rGO (Chatterjee et al. 2014) were found to activate inflammatory response of cells by binding to toll-like receptors (TLRs) and activating the NF-κB signaling pathway. However, further investigation abundance of IL-6 and IL-8 as determined by ELISA indicated an absence of a dose–response relationship (Vranic et al. 2018). Overall, sheets of GO might act as nanotraps for cytokine adsorption, or decreased abundances of cytokines might be due to post-transcriptional regulation.

Genotoxicity

GFNs are capable of producing indirect or secondary genotoxicity. Nano-GO could intercalate the DNA helix between base pairs likely due to their planar structure or sharp edges (Estimation of genomic instability and mutation induction by graphene oxide nanoparticles in mice liver and brain

tissues). Moreover, the H-bonding and π-π stacking may be the dominant forces mediating interactions between AG-QDs and DNA, leading to the DNA chain cleavage (Xu et al. 2018). GFNs have been observed to further down-regulate genes governing DNA repairment, such as RAD51, ATM, PARP1 and base excision repair (BER) signaling pathway genes, implying that GFN likely induce genomic instability (Lu et al. 2017; Sasidharan et al. 2016). Fragmentation of DNA is mainly controlled by endogenous cellular enzymes known as "apoptotic endonucleases". There exists evidence suggesting that graphene treatment in NRK-52E cells leads to increased DNA endonuclease activity through activation of heme oxygenase-1, apoptotic endonucleases and caspase-3. Moreover, caspase independent pathways are involved in DNA fragmentation through elevation of EndoG (Fahmi et al. 2017). Breakage of DNA strands generated by endonuclease activity occurred during the initial stages of cell injury in H9c2 cells after exposure to 60 μg/mL nano-GO (Arbo et al. 2019). Cell cycle arrest has been observed following exposure of lymphocyte cells to GO nanosheets by decreasing the number of cells in the G2/M phase (Babadaei et al. 2018). Inhibition of proliferation was also detected following exposure of hydroxyl-modified GQDs (OH-GQDs) to human esophageal epithelial cell line HET-1 where a significant increase in G0/G1 phase arrest occurred (Li et al. 2018a).

Interactions with proteins

The effect of GFNs on structural integrity of proteins is a major concern due to their affinity for macromolecules. Entrapped biomolecules on the surface of graphene materials might alter the tertiary structure of proteins (Figure S1) (Gu et al. 2019). Previously, effects of nano GO (NGO) sheets on the quaternary structure of human hemoglobin (Hb) near Tyr residues induced α-helicity of Hb in a dose-dependent manner (Babadaei et al. 2018). Interactions of hepcidin peptide and GFNs resulted in formation of stable complexes, resulting in β-sheet structural distortions of peptides and loss of normal functionality, including antibacterial and -fungal activity and iron metabolism (Singh et al. 2018). Another study found that exposure to graphene nanosheets induced blood coagulation, due to resulting instability of blood-coagulation proteins (the tissue factor/FVIIa binary complex) bound to the lipid bilayer membrane (Jo et al. 2017). The hydrophobic property of GO quantum dots (GOQD) was found to increase the surface charge and decreased surface hydrophobicity of the hen egg white lysozymes (HEWL), inhibiting hydrophobic assembly and colloidal stability of the protein (Ban et al. 2018). More importantly, the hydrophobic properties of GFNs may directly interfere with normal functioning of proteins. Graphene nanosheets and GQDs were found to bind calmodulin

(CaM), a dynamic Ca^{2+} binding protein, and suppressed Ca^{2+}-free CaM dynamics (Feng et al. 2017). Evidence indicates potential negative impact of GFNs on the structure and dynamic of key proteins involved in calcium signal transduction in a range of cells.

Epigenetic changes

Impacts on epigenetic regulatory mechanisms, including DNA methylation, histone modification, and small RNA regulation have been observed following exposure to nanomaterials (Djurisic et al. 2015; Lu et al. 2016; Qian et al. 2015). However, limited information is available related to the role of GFN materials. Zhan (Zhao et al. 2016) et al. reported activation of miRNA-360 following exposure to GO and suppression of DNA damage-apoptosis signaling cascade through interfering with the component of CEP-1. Similarly, recent evidence further demonstrates that exposure to GO impacts regulation of cox2 (a biomarker of inflammation) expression in human embryonic kidney cells 293T via triggering physical interactions between the downstream enhancer and the cox2 promoter via p65 and p300 complex-mediated dynamic chromatin looping (Figure S2) (Sun et al. 2018). Overall, these findings suggest the important role of epigenetic regulation in GFN-based nanotoxicity and nano-safety, while more in-depth studies are required.

Modulation of cell death

Generally, mechanisms of GFN toxicity do not occur singly, but in complex and interrelated way ultimately impacting cell survival and normal functioning. To date, mechanisms of cellular death following exposure to GFN include apoptosis, necrosis and autophagy. Previously, graphene treatment of NRK-52E cells for 24 h resulted in upregulation of DNase I and caspase-activated DNase expression, while inhibition of its activity effectively alleviated occurrence of apoptosis (Fahmi et al. 2017). Additional apoptosis mechanisms may be mediated by upregulation of pro-apoptotic genes (p53, p21, Bax, Bak, caspase-3) and downregulation of anti-apoptotic genes (Bcl-2) and the consequent reduction of MMP (Gurunathan et al. 2019b). In comparison, necrotic cell death has been observed following exposure to GO manifested as cell membrane breakage and increased abundances of cytoplasmic vacuoles and nucleolysis (Yang et al. 2019). Evidence also indicated that apoptosis and necrosis could mutually occur at the same time following exposure to GFNs. For instance, increased concentrations of Ca^{2+} in FLG treated cells led to depolarization of the mitochondrial membrane and further induced apoptosis and necrosis in HUVEC cells (Sasidharan et al. 2016). Following laser exposure, GO-Ag induced dose-dependent necrosis/

apoptosis in MCF-7 cells and increased intracellular oxidative stress, including release of singlet oxygen $^1O_2$ and hydroxyl radicals (OH·) (Shaheen et al. 2017). Interestingly, in the osteosarcoma (OSA) cancer cell line MG-63, GO inhibited cell growth by disturbing autophagy (Tang et al. 2018). Further evidence supported that the ROS-NRF2-P62 pathway participated in GO-induced autophagy (Yang et al. 2019). In a previous study conducted by our lab, GO triggered p62-dependent apoptosis through impairment of autophagic flux and lysosomal dysfunction in PC12 cells (Feng et al. 2018). These finding suggest a crosslink between different types of programmed cell death caused by GO nanomaterials.

Cytotoxicity of GFNs have varied mechanisms of toxicity ranging from physical damage, organelle dysfunction to interactions with biomolecules including DNA, RNA and proteins (Fig. 2). Other evidence also suggested impacts on cytoplasmic Ca^{2+} (Sasidharan et al. 2016), and extracellular iron deficiency (Yu et al. 2017). Currently, the literature is insufficient to draw conclusions about the potential hazards of GFNs considering results differ greatly among studies and labs.

Gaps in GFN data

Dose and time dependent toxicity

Treatment dose and length of exposure are primary factors influencing toxicity of nanomaterials. Currently, substantial evidence is available which supports dose-dependent toxic effects of GFNs in a variety of cells, including cancer and non-cancer cells (Gurunathan et al. 2013a, 2015a; Yuan et al. 2017). In general, higher doses of nanomaterials resulted in greater toxicity. Treatment with graphite nanoparticles at 30 μg/mL resulted in significant proliferation inhibition of macrophages, meanwhile cell morphology was abnormal. When dosed with 100 μg/mL, cells demonstrated the greatest percentage of necrosis (Liao et al. 2017). This finding was further confirmed following exposure to GO (Srikanth et al. 2018) or functionalized GO (such as AG-QDs) (Xu et al. 2018). In comparison, the effect of incubation time on GNF cytotoxicity remain controversial. Some studies demonstrated time-dependent graphene toxicity (Lahiani et al. 2017; Nasirzadeh et al. 2019), while other evidence hold the opposite idea (Duan et al. 2017). Note that interactions between cells and GNFs might be modulated by time and exposure in combination. Although high concentrations of GQDs significantly inhibited proliferation of the macrophage NR8383, the downward trend of cell activity was decreasing when the incubation time was extended to 48 h since the cellular uptake of nanoparticles mainly occurred within 24 h after exposure (Xu et al. 2018). These findings
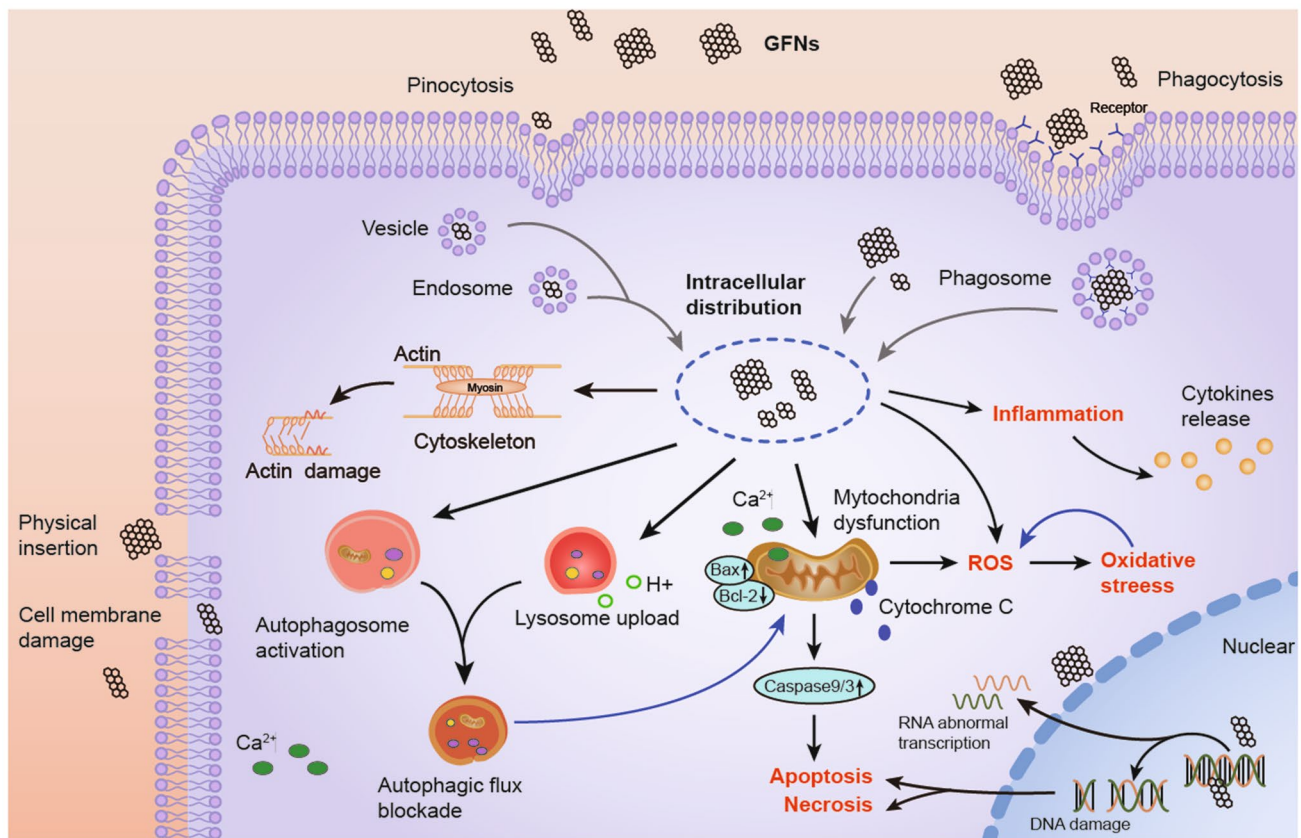
Fig. 2 A schematic diagram revealed the major toxic mechanisms of GFNs to mammal cells. GFNs are internalized into cells via different pathways, further induces varying adverse effects to cell viability, including induction of cell membrane damage and ROS formation, triggering oxidative stress, inflammation and the injury of different cellular components, subsequently leading to apoptosis, necrosis and autophagic cell death

suggested that cytotoxic effects of GFNs have a critical window of exposure. Based on the above-mentioned studies, cells are resistant to GFN toxicity below thresholds of toxicological concern, further supporting their use in biomedical applications.

Particle size

Currently, most studies demonstrated that GFNs of smaller particle size induced greater levels of cytotoxicity. For example, smaller graphene nanomaterials were more easily internalized and consumed greater amounts of intracellular ATP, and produced greater cytotoxicity in caco-2 cells (Saha et al. 2016). At higher doses (50 and 100 μg/mL), 1 layer GO-PEG nanoflake activated greater ROS formation when compared to 4 layer materials, possibly due to greater aggregation of the thinner layer GO-PEG in culture medium (Peruzynska et al. 2017). It should be noted that 1-layer GO-PEG induced greater concentrations of ROS in cell-free medium, indicating potential spontaneous oxidation of medium components which can be eliminated by intracellular catalase

(Gies and Zou 2017). However, another study also indicated that large-sized GO led to more obvious cellular detachment, and greater production of ROS and levels of apoptosis when compared to treatment with smaller-sized GO (s-GO) (Vranic et al. 2018). It is difficult to determine the effect of degree of dispersion and differences in shape on toxicity of GFNs, thus contradictory conclusions may still be drawn from the same cell line treated in different laboratories. The effect of size on GNF toxicity has frequently been investigated in non-phagocytes and adherent cells such as fibroblasts or epithelial cells (An et al. 2018; Lasocka et al. 2018; Saliev et al. 2019; Wu et al. 2018b). Note that the size dependent toxicity of GO flakes might not be applicable to phagocytes, and non-phagocytic suspension cells (Gies and Zou 2017; Yue et al. 2012).

Surface structure

Graphene-based materials have differing surface oxidation states. GO tends to be more easily internalized by cells due to hydrophilicity, while rGO has greater hydrophobicity

as demonstrated by adsorption and aggregation at the cell surface with limited internalization (Chatterjee et al. 2014). Further evidence indicated that the oxygen content and presence of oxygen containing functional groups were involved in GFN-induced cytotoxicity, showing greater toxicity with lesser C:O ratios (Das et al. 2013). Carbon radical content is another contributing factor to the hazard of GFNs. Hydrated GO (hGO) possessing greater carbon radical density caused greater cell death via lipid peroxidation of surface membranes and inducing membrane lysis compared with pristine GO and rGO (Li et al. 2018b). However, a recent study revealed that GO was the least toxic to other graphene-based materials, which was attributed to its smoother edges and regular structure whereas rGO and graphene have more sharp edges and irregularities in shape (Gies and Zou 2017). This apparent discrepancy in GFN toxicity, may be a result of differences in experimental materials and application methods. Note that structural defects in nanomaterials can exert potential impact on their biological effects. When compared to ideal graphene, defective graphene triggered more severe protein denaturation due to stronger attractions of surface residues of HP35 from defect edges (Gu et al. 2019).

Functionalization

Surface modifications have been widely used in modulating the chemical properties (such as functional groups, carbon/oxygen ratio, and hydrophobicity) of GFNs, which are crucial determinants of their biocompatibility. It has been reported that PEGylated GO (PEG-GO) had lesser cytotoxicity when compared to pristine GO after 24 h exposure in A549 cells (Duan et al. 2017). On the other hand, PEG-GO nanosheets with greater levels of oxidation had greater cytotoxicity when compared to those with lower levels of oxidation, further highlighting the important role of oxidation groups related to GNF toxicity (Wu et al. 2018b). Interestingly, PEGylated rGO was detected to cause more severe effect and rates of cellular death caused by oxidative stress in cells of the blood–brain barrier, including astrocytes and RBEC cells when compared to pristine rGO (Mendonca et al. 2016a). These findings further highlight the varying roles of functional group modification in different types of GFNs.

Discrepancies of surface chemistries also participated in controlling GFN toxicity. Compared to surface-modified GO by the PAA polymer showing minimal effects on chromatin structure, aminated GO (GO-NH$_2$) dynamically altered chromatin architecture by mediating epigenetic changes at the cox2 locus (Sun et al. 2018). Under the same treatment dose (100 μg/mL) of COOH-GQDs and NH$_2$-GQDs, OH-GQDs resulted in the greatest rate of cell apoptosis. Analyse of protein expressions demonstrated that all GQDs participated

in regulation of MAPK and Akt pathways, but the expression of p-ERK1/2 and p-JNK varied greatly among nanomaterials (Xie et al. 2019). Additionally, diverse compositions of the protein corona, especially immunoglobulin G (IgG) formed on their surfaces may be responsible for biocompatibility diversity. IgG within the protein corona could be readily recognized by immune cells such as macrophages, further determining biological behaviors of pristine GO and its derivatives, especially on interactions with cell membrane and cellular uptake, leading to thrombus formation in blood (Xu et al. 2016).

Protein adsorption

Based on their high surface free energy, nanomaterials are rapidly coated by proteins in biological matrices to form a so-called "protein corona". An important constituent of protein coronas, opsonin (such as immunoglobulin G), may help identification of nanomaterials by immune cells and uptake by the reticuloendothelial system (RES) (Aggarwal et al. 2009). Reorganization of protein coronas can impact biological behavior of nanomaterials. Hydrophilic interactions of GO in culture medium results in formation of hard protein coronas (enriched in FBS proteins) involved in regulation of multiple biological pathways including cellular development/structure, lipid metabolic processes, and signal transduction (Franqui et al. 2019). Additionally, nontoxic PEG-GO become more toxic when exposed to heme-containing proteins, including lactoferrin, transferrin and ferritin due to enhanced peroxidase-like activity (Zhang et al. 2017). A recent study also indicated that large sized-GO (l-GO) triggered greater cytotoxicity following incubation in the presence (w/FBS) of 10% FBS when compared to those without FBS (w/o FBS). In contrast, FBS treatment on small sized-GO (s-GO) demonstrated the opposite effect on cell monolayer. (Vranic et al. 2018). Additional evidence also highlighted the protective role of protein coatings against GO-induced cytotoxicity (Duan et al. 2015; Vranic et al. 2017).

Protein adsorption by GFNs is linked to their dimensionality. Generally, 2D nanomaterials, such as graphene and graphene oxide flakes, may provide a better surface for anchoring protein residues, thus facilitating the adsorption of proteins and maintaining their structure. Planar graphene oxide flakes have higher capabilities to sequester and adsorb proteins in comparison with cylindrical shaped MWCNT (Pastrana et al. 2019). This observation was confirmed by the molecular dynamics simulation, which observed reduced π–π stacking with aromatic residues of proteins in cylindrical nanostructures and limited adsorption (Gu et al. 2015). Whether GFNs can be internalized into cells is controversial, thus a lower availability of media

protein can be considered as a major cause of cell toxicity induced by these nanomaterials.

Impurities and mixture effects

Intracellular uptake of graphene by macrophages may increase in the presence of endotoxin. It was detected that response of cells to graphene exposure resulted in different expression of genes in the Toll-like receptor pathway, NOD-like receptor pathway and downstream signaling molecules when treated with endotoxin (Lahiani et al. 2017). Thus it is reasonable to conclude that the removal of endotoxin or other bacterial contaminants is essential for biosafety evaluations of nanomaterials (Li et al. 2017; Mukherjee et al. 2016). Moreover, mixture of nanomaterials with other chemical components might affect their biological interactions. Traditionally prepared GO materials often include high concentrations of Fe^{2+} and Mn^{2+}, which exhibit high mutagenicity to cells (Ou et al. 2016; Peng et al. 2015). Additionally, pristine GO and GO-Ag nanocomposites under the same dose demonstrated different immunotoxicities after 24 h exposure in J774 macrophages, where GO-Ag induced a greater proportion of macrophages to undergo late apoptosis/necrosis while pristine GO mainly induced early apoptosis (de Luna et al. 2019).

Perspectives and challenges

Currently, the evidence is insufficient to draw conclusions about potential hazards of GFNs. Inconsistencies in toxicological data of GFNs are likely due to different physicochemical properties (Gies and Zou 2017; Li et al. 2018b), assay (Dziewięcka et al. 2017; Lee et al. 2019; Petibone et al. 2017; Syama et al. 2017; Tang et al. 2018), and varying experimental conditions (Gurunathan et al. 2015a; Sasidharan et al. 2016; Yuan et al. 2017). Based on existing evidence, we believe special attention should be paid to the sections outlined below and would help improve biocompatibility assessments.

Manufacturing technology

Currently there is consensus that physicochemical properties of nanomaterials (such as size, chemical composition, surface functionalization and hydrophobicity) impact how they interact with the biological environment. Thus, accurate characterization of GFN physicochemical properties is indispensable in all future toxicity studies. Although, a growing number of studies have revealed relatively detailed characterization, remarkable discrepancies still exist which may result from differing manufacturing techniques leading to significant differences in GFN properties (Gurunathan et al. 2019a; Li et al. 2018b; Nasirzadeh et al. 2019). In the majority of studies, experimental materials are prepared in-house, which can further complicate the comparison of results. Thus, a universal method is required to facilitate better comparison of data among labs.

Conditions of dispersion

When compared to the experimental conditions of dose, time, and particle size of GFNs, degree of dispersion of nanomaterials is more difficult to control. For instance, AG-QDs are uniform particles, corresponding to a single layer of oxidized graphene with an average lateral size of 4.1 nm and thickness of 0.72 nm. After incubation in culture medium for 24 h, both lateral size and thickness of individual AG-QDs increased to nearly 10 nm, indicating substantial adsorption of medium components such as FBS (Xu et al. 2018). The observed aggregation behavior in biological matrices also occurs as GFN tends to form large aggregates rather than individual units in liquids. Although the thickness of these nanomaterials is generally at the nanoscale range, the lateral size of graphene sheets can range from several nanometers to micrometers following formation of agglomerates in liquids (Hinzmann et al. 2014). The stability and dispersal of nanomaterials impacts their observed toxicity. When aggregation of GFNs occurs, the surface area and are available for contact is significantly reduced (Pavlin and Bregar 2012). Therefore nanomaterials should be used quickly to ensure uniform dispersion and to prevent excessive agglomeration in liquids. Currently, for carbon-based nanomaterials, including GFNs, ultrasonic mechanical energy is commonly used with the addition of organic dispersants to obtain homogeneous dispersion (Maktedar et al. 2017; Pattammattel et al. 2017; Zhang et al. 2018). Nevertheless, strong mechanical forces may alter the physical properties of nanomaterials, by inducing fragmentation and resulting in defects on the material surface or edges, consequently altering their interactions with biological systems (Gies and Zou 2017). Moreover, the stability of ultrasonically dispersed nanomaterials is poor. As a result, proper control of experimental conditions to facilitate improved comparisons has become a topic of interest. A recent study demonstrated a more efficient method to ensure consistency among experiments using rapid (ultra-turrax, UT) mixing to regulate the formation of protein corona and reduce GO agglomeration in the presence of proteins, which allows for more efficient cellular uptake with limited cytotoxicity (Reina et al. 2019). We believe that the use of UT protocol will promote the preparation of next-generation GO-based drug-delivery platforms.

Detection methods

Different detection methods, including observation criteria, parameters, and selection of testing methods may generate large inter-laboratory differentiations (Ema et al. 2012, 2014). Evaluation of GFN exposure is a key step in developing a better understanding of their cytotoxicity and underlying mechanisms. The most commonly used detection methods include direct observation of localization of nanomaterials in cells by TEM (Contreras-Torres et al. 2017), or quantitative analysis of nanomaterials by fluorescent or radioactive labeling (Li et al. 2018b; Ma et al. 2015). Additionally, changes in side-scatter (SSC) and forward scatter (FSC) characteristics via flow cytometry might correlate with particle uptake since intracellular density increases when NPs enter the cell (Babadaei et al. 2018; Contreras-Torres et al. 2017; Xu et al. 2019). However, traditional methods of detection have shortcomings such as low observation efficiency, large error of quantitative results, and can have harmful effects on nanoparticles. A recent study in 2019 reported a novel gel-electrophoresis based method which could be used for accurate quantification of GO in cell samples, with a detection limit of 84.1 ng and which is applicable in a number of different cell types (Xin and Wan 2019).

Another important consideration is the potential for interference between nanomaterials and cytotoxicity test reagents. It has been demonstrated that results of the MTT test may not be suitable for assessment of cell viability since GFNs endocytosis or cell membrane adhesion by living cells would interfere with absorbance readings (Gies and Zou 2017). In comparison, the WST-8 assay has more reproducible and reliable results, and does not require intensive sample preparation especially for treatments receiving high concentrations of GO. Similar findings have been observed when applying the AB and Neutral Red assays due to fluorescence quenching or direct interferences (Monasterio et al. 2017; Srikanth et al. 2018; Talukdar et al. 2014). Due to limitations of traditional cell viability assays, results should be carefully interpreted.

Resistance and biodegradation

Upon exposure to GNFs, cells could develop resistance to these harmful stimulus by reducing the uptake of nanomaterials (Xu et al. 2018), or excreting internalized nanomaterials via lysosome secretion, vesicle-related secretion, and non-vesicle-related secretion (Gurunathan et al. 2019b). Once nanomaterials are inside the cells, how effectively they can be degraded or excreted becomes critical. Existing evidence support that carbon-based nanomaterials are degradable/biodegradable through the photo-Fenton reaction, which was able to oxidize GO flakes into individual pieces known as GQDs (Bai et al. 2014; Kotchey et al. 2011). Another

in vivo study also suggested the possible biodegradation of graphene and highlighted the important role of macrophages during the degradation process. (Girish et al. 2013). Moreover, Kurapati et al. (2015) discovered the important roles of hydrophilicity, surface charge, and colloidal stability of the aqueous GO in their biodegradation by myeloperoxidase catalysis, which was derived from human neutrophils. Note that intracellular autophagosomes and lysosomes are effective approaches for the degradation of foreign bodies. However, these defense mechanisms may aggravate cell damage through blockage of autophagy flows and lysosome membrane permeabilization (LMP) due to the special physiochemical properties of GFNs (Feng et al. 2018; Kalman et al. 2019). Collectively, we believe further studies should pay more attention to the detailed mechanism of GFN degradation and the methods for enhanced degradation effects.

Summary

This review has summarized recent progress towards an understanding of the biological and environmental hazards posed by GFNs. The first step is to "know the materials" with a detailed description of the characteristics of GFNs and their biomedical applications. Secondly, both their in vivo and in vitro activity and mechanisms of action contributing to the observed adverse effects need to collected and analyzed. Moreover, influencing factors and data gaps are equally important in improving risk assessments. However, current biosafety assessments of nanomaterials cannot reach a comprehensive conclusion due to the lack of reliable experimental models, effective detection techniques and recognized evaluation standards. We hope the current literature survey can serve as an important step to systematically collect biosafety data of GFNs and further promote their application.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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