Review

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Graphene-based Nanomaterials: Uses, Environmental Fate, and Human Health Hazards

Heidi N du Preez[1,2], Matthew Halma[3]

[1] Catalysis and Peptide Research Unit, University of KwaZulu-Natal, Westville Campus, Durban 4041, South Africa
[2] College of Health Sciences, University of KwaZulu-Natal, Durban 4001, South Africa
[3] EbMC Squared CIC, Bath BA2 4BL, UK

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Abstract

Graphene-based materials (GBMs) possess remarkable physiochemical properties, making them promising for diverse applications in biomedicine, agriculture, food, and industrial applications. Human and environmental exposure to GBMs is increasing at an unprecedented rate, yet there is still a knowledge gap regarding the safety of GBMs. This review summarizes the physiochemical properties of GBMs and critically examines the possible effects of GBMs, both at the level of molecular mechanism and at the level of the organism. While oxidative stress-mediated cell damage has been proposed as a primary cytotoxicity mechanism for GBMs, various *in vivo* biodistribution and cytotoxicity mechanisms are also highlighted. This review of the literature provides an overview of the cytotoxicity of GBMs, raising concerns about their widespread application with potential hazardous consequences on the environment and in human health.

Keywords: nanotechnology; graphene; graphene oxide; cytotoxicity

Introduction

Nanotechnology is a very fast growing industry, with the main market prospects being the use of engineered nanoparticles (NPs) for medicine, food, agriculture, computers and conductors [1], causing increased human exposure. Nanomedicine is the application of nanomaterials in medicine, which are used in vectors, biosensors, diagnostics, and drug and gene delivery [2, 3]. Nanomaterials consist of NPs with dimensions less than 100 nm [4]. Nanomaterials, such as metals, nonmetals, metal oxides, lipids, and polymers, are scientifically engineered for various applications. The emergence of nanomaterials in recent years has rapidly transformed the scientific landscape in fields as diverse as aerospace, military, and medicine [5].

Graphene and graphene-based materials (GBMs) for healthcare applications are among the fastest growing fields of science and technology. As the thinnest, strongest, and stiffest material, virtually indestructible, graphene and its chemical derivatives are a form of carbon in a very thin monolayer atomic sheet, arranged in a 2D honeycomb lattice, with small lateral dimensions and a large surface area [6, 7]. Graphene-based nanomaterials (GBNs) exhibit unique antibacterial and antiviral properties, because of their small size, large surface area, targetability,

and stimulus-responsive characteristics.

Various reviews and research studies have appeared in the scientific literature on the possible contribution of GBNs as theranostic agents in the global fight against COVID-19 [5, 8–10]. Graphene-based textiles, air filter systems, personal protective equipment (PPE), face masks, hand sanitizers, vaccines, antiviral surfaces and coatings have been proposed, or developed, to control the epidemiological spread of COVID-19, as well as to develop environmental biosensors and other diagnostic techniques [4, 8, 11–14]. Furthermore, various NPs, such as iron, zinc, copper oxide, silver, and GBNs, are added to DNA and mRNA delivery systems in preclinical research due to their unique antiviral properties [4, 7, 15]. Due to its unique physiochemical properties, graphene oxide (GO) is an attractive and popular material for DNA and RNA delivery and detection, where its interactions are based on electrostatic forces and π-π stacking [16]. GBNs have been proposed to be engineered to directly target SARS-CoV-2 [8, 14]. GBNs have also been used for extracorporeal perfusion of cytokines from the blood circulation to prevent sepsis [5].

Unfortunately, even at very low doses, GBNs can have significant harmful biological impacts that occur through multiple mechanisms, with their cytotoxicity profile highly unpredictable [17–21]. Therefore, nanomedicine may be seen as a double-edged sword. Although there are many concerns about possible toxicity and increased risk of particle aggregation, GBNs have proven to be effective in improving the efficacy of many drugs; act as a nanodrug to inhibit viral attachment, fusion, replication, and infection; or they can suppress the pro-inflammatory cascade following viral infection [22]. Nonetheless, there are several reports about GBNs that are associated with mutagenicity, tumorgenicity, free radical production, and penetration into the brain [22]. The toxicity would be related to the size and dose of the NPs, the route of administration, biodistribution, and biodegradability [5]. More research is needed to seriously assess the potential toxicological effects of GBNs, and NPs in general [23]. According to a 2018 review, only 250 (1.3%) out of a total of 19 000 publications on GBMs reported any toxicity data, while only 70 (0.4% of total) included *in vivo* toxicity data [24]. Furthermore, large-scale sophisticated production processes and intellectual property rights of GBNs can increase their price and conceal their widespread usage [1].

In this review, we summarize the physiochemical properties of GBMs and our current understanding of their cytotoxicity mechanisms. The aim is to provide an overview of the cytotoxicity of GBMs and to raise concerns about their widespread application with potential hazardous effects on the environment and human health.

Graphene-based Materials

Carbon forms many allotropes, with the major ones being graphite and diamond. Graphene is extracted from graphite using a technique called micromechanical cleavage [25]. GBNs are synthesized through diverse methods, encompassing covalent and non-covalent approaches, chemical deposition, hydrothermal growth, electrophoresis deposition, and physical deposition [26]. GBMs could include monolayer graphene, few-layer graphene, ultrathin graphite, graphene quantum dots, graphene nanosheets (GNSs) and graphene nanoribbons [2, 27]. The variety of GBN morphologies used in biomedicine, as well as their doped and functionalized derivatives have been described in a recent review [28].

GBNs are not homogeneous and vary in number, lateral dimension, surface chemistry, defect density, or quality of individual graphene sheets, composition and purity [29]. The planar system of graphene exhibits unique physiochemical properties, predominantly related to its high electronic and thermal conductivity [30]. To improve mechanical strength, GBNs are ideal nanofillers for polymeric hydrogels, due to their large surface area, flat structure, water dispersibility and biocompatibility, in addition to the intrinsically excellent thermal stability, thermal and electrical conductivity, optical, magnetic, electrochemical, photothermal, photoluminescent, and mechanical properties [6, 31, 32].

These superior properties make graphene and its derivatives ideal for many biomedical applications, such as anticancer therapy, nanomedicine, drug, gene and protein delivery, antimicrobial agents, biological imaging, molecular biosensors, bioengineering, biotechnology, organic electronics, memory

applications, and tissue engineering [2, 3, 33, 34] (Fig. 1).

Nanographene oxide (nGO) is the most popular graphene-based nanofiller due to its good dispersion capacity in water, which is a vital factor for the construction of hydrogels [34]. GO is obtained from graphite powder by oxidative exfoliation using strong oxidants and acids. Due to these harsh synthetic procedures, GO possesses defects in its hybridized sp^2 orbitals, with consequently diverse functional groups, such as epoxy, phenolic, hydroxyl, carbonyl, and carboxylic groups. These oxygen-containing functional groups of GO render it highly reactive with reduced size, while aiding its dispersion in aqueous solutions and miscibility with hydrophilic polymer chains in hydrogels, making it ideal for drug and gene delivery or tissue engineering [35]. GO exhibits hydrophilic edges with oxygenated functional groups and a hydrophobic basal plane, enabling its multiple molecular interactions with various molecules, optimizing drug and gene loading [7].

Therefore, the main reasons for adding functionalized GO, such as polyethylene glycol (PEG)-nGO, to hydrogels would be their reliable aqueous dispersibility and colloidal stability, enhanced molecular adsorption, increased gene/drug loading capacity, allowing sustained drug release, and improved electrical and thermal conductivity [7, 34, 36], in addition to their efficacy against various microbes and viruses [8, 15]. nGO is often modified with PEG to improve mechanical properties and stability [16, 31, 34].

Although GBNs have been used for many biomedical applications, their biocompatibility and toxicity remain controversial, affecting clinical translation [27, 36, 37]. Potential cytotoxicity and biocompatibility of GBNs are determined by factors such as surface area and charge, layer number, shape, dosage, morphology, lateral dimension, stiffness, synthetic synthesis method, surface functionalization, surface chemistry, purity, aggregations, dispersion state, exposure route and time, oxidative state of the host, cell-type specific, protein adsorption and experimental setup [7, 9, 29, 33, 34, 36] (Fig. 2).

With the widespread application of GBMs in various industries, their possible toxicological effects

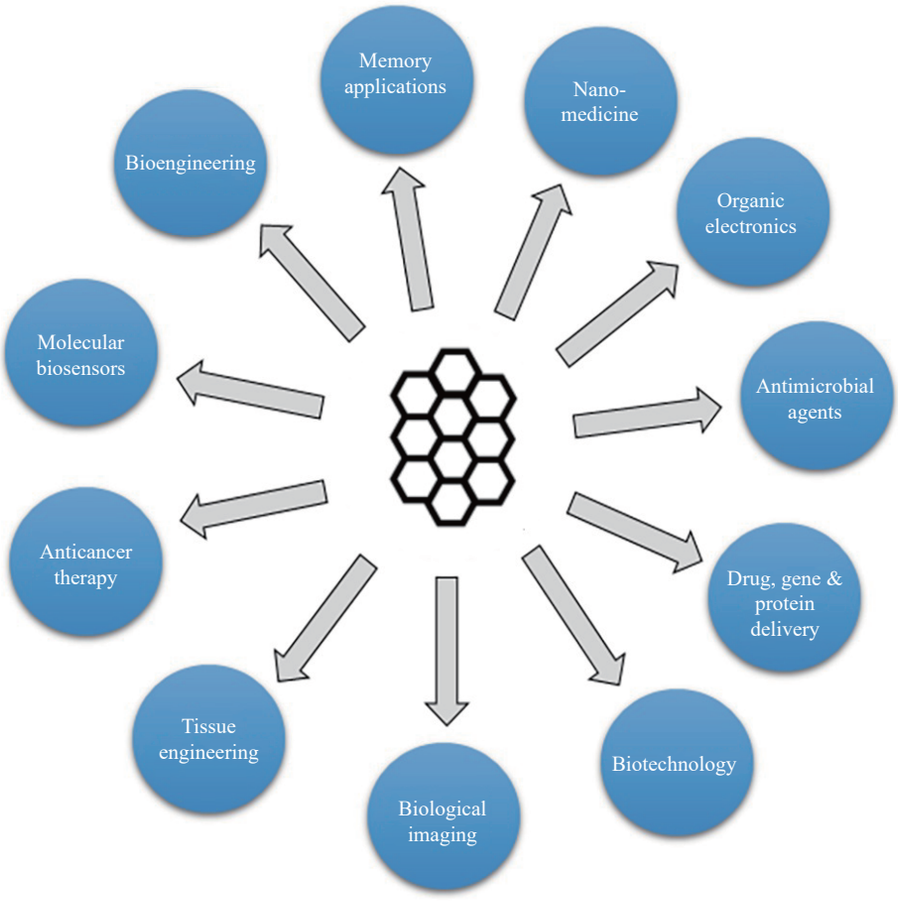

Fig. 1 The various biomedical applications of graphene-based materials.

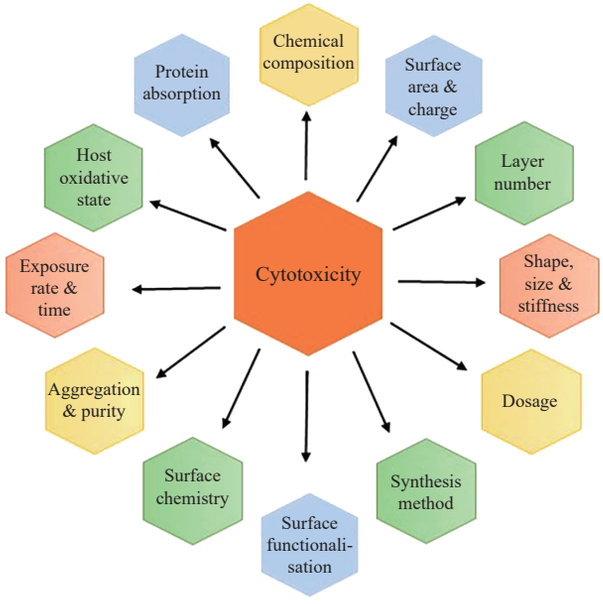

Fig. 2 Various factors determine the potential cytotoxicity and biocompatibility of graphene-based nanomaterials.

need serious consideration, as reviewed in the following section.

Safety Concerns

Some of the known underlying toxicity mechanisms of GBNs include physical destruction, oxidative stress, DNA damage, inflammatory responses, bioaccumulation, apoptosis, autophagy, and necrosis [27]. Graphene nanoribbons can mechanically damage cell membranes, stimulate the production of reactive oxygen species (ROS), fragment DNA and produce aberrations of chromosomes (Fig. 3) [27, 37]. Administration of PEG-nGO can cause tissue destruction and promote various diseases, such as atherosclerosis, rheumatoid arthritis, heart disease, cancer, and neurodegenerative diseases, while GBN-induced ROS could cause oxidative stress in multiple organs, for example the brain, heart, and kidneys [38].

The debate continues on the possible toxicological effects of GBNs, where some studies indicate no risk, while others confirmed that GBNs are cytotoxic and may cause adverse effects in exposed individuals [27, 39–41]. There are several reasons for the conflicting results obtained from the safety studies. Although the section below mainly focuses on GBNs, it mostly applies to NPs in general.

(1) GO is not a single structure with fixed properties. Synthesis, manufacturing, and functionalization result in great variability in the morphological characteristics and physiochemical properties of GBN end products, which can contain unreacted and residual chemicals, metals, and other

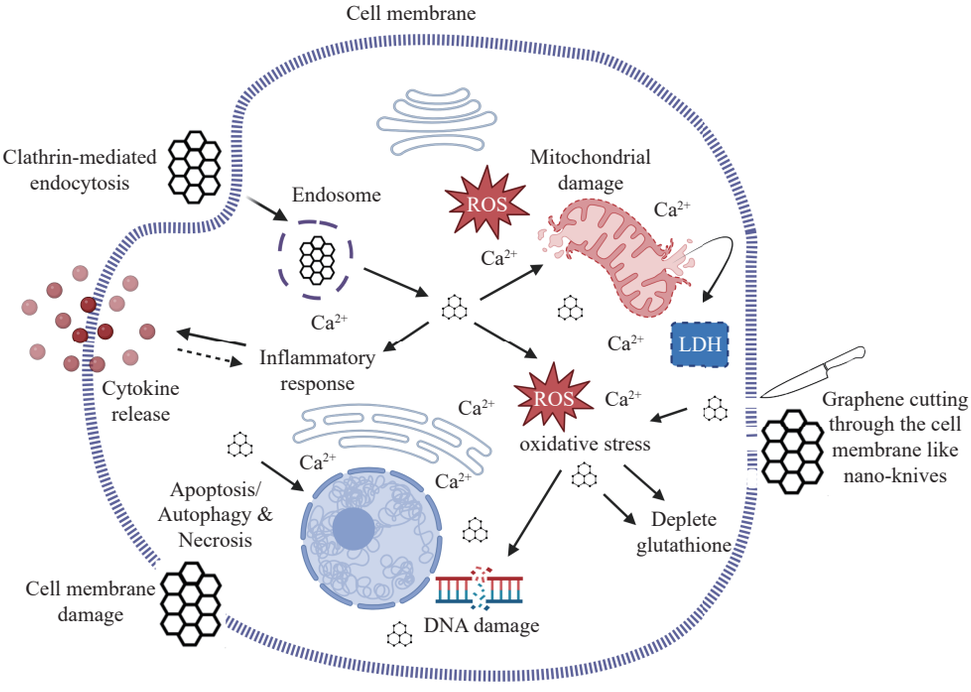

Fig. 3 Schematic representation of the possible mechanisms of GBN cytotoxicity. GBNs can enter cells through physical destruction of the cell membrane, clathrin-mediated endocytosis or phagocytosis. The internalized GBNs can result in reactive oxygen species (ROS) generation, glutathione (GSH) destruction and depletion, release of lactose dehydrogenase (LDH) and free calcium ions (Ca^{2+}). Subsequently, GBNs result in cell injury through oxidative stress, inflammatory responses, DNA and mitochondrial damage, with consequent apoptosis, autophagy and necrosis.

impurities, apart from carbon [27, 29, 35, 42]. GO contaminated with manganese is known to be cytotoxic, by reducing metabolic activity and causing membrane injury, while graphene prepared by Hummer's method may contain chlorate and nitrate anions, also affecting its physiological and toxicological properties [43].

(2) *In silico* or *in vitro* studies cannot be accurately extrapolated to the reality that *in vivo*. Graphene oxide nanoparticles (GONPs) can interact with proteins and change their conformation and activity, and they can also interact with reagents used in toxicity tests, even though some studies do not show any dependence on culture medium. It could be expected that interaction with cell media can change the stability of GONPs and its transport properties, when determining their biodistribution in living organisms [43]. Most *in vitro* toxicological studies have focused on direct interactions between nanomaterials and biological components or cells, while entrapment of GO in hydrogel matrices would minimize the prompt and direct toxic effects of GO *in vivo* on cells [7]. Furthermore, analysis and conclusions obtained under static *in vitro* conditions cannot be accurately translated directly to infer the hematological properties of GBMs in dynamic *in vivo* environments [42]. In addition, the culture medium used in *in vitro* studies cannot compare with the complex *in vivo* reality, where various enzymes, amino acids, vitamins, and minerals facilitate the plethora gene and signaling reactions.

(3) The different research groups and diverse cellular or animal models used, as well as variation in the GBMs used.

(4) Different ways of exposure, administration and entry paths of GBNs, different tissue distribution and excretion, as well as various cell uptake patterns and locations [27].

(5) There are no current standardized methods for assessing the immunotoxicity of NPs on the immune system [39].

(6) GBMs in colloid form may interact with physiological media resulting in aggregation and flocculation of the suspension [29].

(7) The physical interaction of GBNs with cell membranes is one of the major causes of graphene cytotoxicity. Differences in cell surface charge will determine whether hydrophilic GO is internalized by nonphagocytic cells or not [27]. An intact, negatively charged sulfated glycocalyx (GL) would repel hydrophilic GO. However, during inflammation and reduced sulfation, shedding of the GL components could be expected, with more available cationic binding sites exposed on the cell surface, enabling NPs to be more easily taken up by scavenger receptors. Most of the GBNs and graphene safety studies mainly consider the hydrophobicity of the cell membrane, but do not take into account the effect of the negatively charged intact sulfated GL, and the phenomenon of undersulfation, or a degraded GL due to the shedding of glycosaminoglycans, especially during inflammatory conditions [44].

(8) In addition to the hydrophobicity of GBNs that plays a role in its interaction with the lipid bilayer of cells, surface energy may be modified *in vivo* by the formation of a protein corona on the surface, modifying the membrane response to GBNs [34].

(9) A distinction should be drawn between scenarios where mechanical stress or shear forces may be an additional factor to cell destruction, compared to spontaneous membrane incorporation alone [34].

(10) Most studies, such as hemocompatibility and hemotoxicity assessments, have been carried out only over a short period. Therefore, the long-term *in vivo* hematological effects of GBNs are still largely unknown or poorly understood [42]. Furthermore, NP metabolism and excretion of NPs are long-term processes and the long-term accumulation and toxicity of GBNs in different tissues remain unknown [27].

(11) Since GO is not a distinct structure with static properties [35], it makes standardization of research challenging. Although many investigations are not directly comparable, there is an urgent need for standardized protocols and systematic approaches to assess the biocompatibility and cytotoxicity of GBMs [42].

Furthermore, it is not clear whether conflicting reports regarding the interaction of GBNs with cells are due to the specific NPs used, synthesis residues, or exposure conditions. Most GBNs are difficult to label with fluorescent dyes, or to discern in TEM and quench fluorescence, making uptake studies challenging. GBNs must first be coated with protein to be visible in confocal microscopes. Therefore, uptake is reported to depend on surface coating,

particle size, cell type, and exposure time. Transmission electron microscopy (TEM) images may show particles in the cytoplasm and small amounts in the cell nucleus, as well as signs of autophagy. Currently, confocal Raman spectroscopy seems to be the most promising method for the detection and measurement in cells, allowing the detection of very low concentrations of GBNs [43]. At higher concentrations, graphene can be imaged, owing to its intrinsic photoluminescence properties [16, 36, 45, 46].

Cytotoxicity of Graphene-based Materials

Lalwani et al. and Ou et al. extensively reviewed the cytotoxicity of GBMs [27, 37]. Yet, several *in vitro* and *in vivo* studies have been performed over the years to assess the potential risks of GBMs, and NPs in general.

Adsorption and distribution

GBNs can enter the human body through inhalation, ingestion, dermal penetration, injection, or implantation for biomedical applications [34]. The extent and severity of GBN-caused toxicity would depend mainly on the route of entry and the duration of exposure in the human body [29]. Due to their small size, GBNs can enter organs by crossing various barrier systems, such as the blood-air barrier, blood-testis barrier, blood-brain barrier, and blood–placental barrier [27, 29].

It has been suggested that protein coated GBNs with different sizes are taken up by distinct mechanisms. It has been observed that carbon nanotubes and functionalized graphene flakes passed through the cell membrane and accumulated in the region of the perinuclear, without any observation of membrane destabilization [47, 48]. Graphene quantum dots probably penetrate cell membranes directly, rather than through energy-dependent pathways [41, 49]. Where small particles are taken up mainly by clathrin-mediated endocytosis (Fig. 3), larger NPs and protein-coated GONPs are internalized by a combination of both clathrin-mediated endocytosis and phagocytosis [27, 36, 43].

Membrane and cellular damage

The antimicrobial properties of GBNs can be attributed to ROS generation, damage to pathogen cell membranes, and interference with microbial or viral metabolic activity [50]. Potent hydrophobic interactions of GBNs with cell membranes can result in the morphological extension of the filopodial and cytoskeletal dysfunction of F-actin. GO and its derivatives can dramatically decrease the expression of differential genes responsible for structure and function, such as regulation of the actin cytoskeleton, focal adhesion, and endocytosis [27]. Furthermore, the size of the graphene flake and the degree of GL sulfation of the pathogen membrane were shown to influence the antimicrobial activity of functionalized graphene materials [9].

The intrinsic antimicrobial properties of GBNs have been ascribed to the fact that they act as nano-knives due to their sharp edges, leading to physical destruction of the lipid bilayer and oxidative stress, and direct adhesion to bacterial or viral membranes (Fig. 3) [5, 7, 27–29, 50]. Therefore, the sharpened edges of GBNs can act as ‘blades’, inserting and cutting through cell membranes [51]. Therefore, the basis for GO-induced cytotoxicity is attributed to the cutting and extraction of membrane phospholipid chains from cell membranes [52]. The loss of integrity of the bacterial membrane intensifies with increasing concentrations of GO, confirmed by increased release of lactose dehydrogenase in culture medium (Fig. 3) [53]. However, if GBNs have such a destructive effect on microbial cells, these same toxic effects can be expected in mammalian eukaryotic cells [7]. In murine studies, macrophages and neutrophils have been found to undergo unusual morphological changes upon contact with GONPs [27, 54]. After being internalized, GO caused cytotoxicity in macrophages by accumulating in the cell cytoplasm, perinuclear space, and nucleus. This was achieved by increasing intracellular ROS, depleting the mitochondrial membrane potential, and activating the mitochondrial apoptosis pathway (Fig. 3) [27].

In addition, GO has been investigated mainly for biosensing, as a matrix to immobilize several enzymes, such as glucose oxidase, horseradish peroxidase, and hemoglobin. GO was demonstrated to inhibit the activity of chymotrypsin [55]. Therefore, GBMs may up- or down-regulate, or inhibit, various enzymes, affecting their activity and/or stability. Kaloudis et al. demonstrated that less negatively

charged enzymes are more likely to be deactivated through their interaction with GO [56]. The specific effects that GBNs may have on active biomolecules, such as enzymes and proteins, need further research.

Oxidative stress

GBN-induced oxidative stress is one of the main causes of cytotoxicity, which can be attributed to GBN-cellular interactions and ROS generation (Fig. 3) [37]. It was demonstrated that even at very low concentrations, GONPs can generate ROS [43]. After exposure to GONPs, the activity of SOD and glutathione (GSH) peroxidases decreased in a dose-dependent manner [27]. As with mercury, GO has a high affinity for sulfur, or SH groups in molecules, during the redox process, acting as a catalyst in oxidative desulfurization reactions and subsequently affecting many cellular processes [57, 58].

Moreover, ROS levels will deplete GSH, in addition to GSH being deactivated by GO (Fig. 3) [34, 59]. There is also the possibility that GO can transform to reduced GO (rGO) *in vivo* through direct interaction with organisms, oxygen, and other biomolecules [29].

Inflammation

Through triggered inflammatory responses and ROS, GBNs can cause cell death through autophagy and necrosis, as well as apoptosis through damage to the plasma membrane (Fig. 3) [27, 29, 60]. It was shown that rGO caused apoptosis even at low doses and at an early time point, triggered by the death receptor and the canonical mitochondrial pathway. Ma et al. demonstrated that GO can bind to toll-like receptors (TLRs) and activate the NF-κB signaling pathway in cells [37, 61], where the autophagy pathway is related to phagocytosis by TLR signaling in macrophages [62].

Algadi et al. found that GONPs caused up-regulation of IL-6, macrophage inflammatory protein (MIP)-1α, MIP-1β and MIP-2, at much lower concentrations of GONPs than those required to cause cytotoxicity (Fig. 3) [39]. It has also been shown that in primary and immortalized macrophages, GONPs may stimulate the secretion of Th1/Th2 cytokines, such as IL-1a, IL-6, IL-10, TNFα, and granulocyte-macrophage colony-stimulating factor, as well as chemokines such as MCP-1, MIP-1a, MIP-1β, and RANTES [63, 64]. While cytokines mainly modulate macrophage functions and cell surface marker expression (autocrine effects), chemokines play a role in recruiting circulating monocytes to tissues (paracrine and endocrine effects) [65].

Aggregation

GBNs have an intrinsic tendency to aggregate [28]. They may initiate protein aggregation at physiological pH, causing the induction of unfolded, amorphous protein–NP complexes, followed by large protein clusters. The conformational changes of proteins initiated by GBNs are very likely to further catalyze the formation of aggregated species and their extension [66]. To prevent the aggregation of GNSs in the aqueous phase, GO and iron oxides are combined to form magnetic GO [3].

The magnetic properties of GO per se are very diverse, since the atomic composition of GO is not stoichiometric and one can expect sample-to-sample variability, depending on the starting graphite material and method of production, the presence of other metals and the degree of oxidation [67]. The magnetic potential of NPs is important. Uniquely formulated magnetic NPs can be guided through the body through a system of external magnets to facilitate increased drug concentration, for example, in the tumor environment [68].

Environmental exposure

In biomedicine, there are a broad scope and numerous advantages of using GBNs in many different applications, such as cancer detection, photonics or plasmonics, electronics, sensors, catalysis, drug and gene delivery, controlled stem cells, and DNA sequencing or CRISPR technology [8, 28, 36, 39, 69]. However, the possible cytotoxic and genotoxic effects on humans and the environment need serious consideration, and more research is needed before safe clinical application of these materials would be possible. When nanotechnology is used as the delivery vehicle for drugs and genes, the side effects on normal healthy cells, other than the target cells, should be considered. GBN interactions have been demonstrated with various essential biological molecules, including small molecules and ion adsorption, DNA and RNA interactions, catalysis of oxidative reactions, as well as protein and lipid interactions [27].

GBNs have the potential for widespread human

exposure, as they are used not only in the medical sciences, but also in agriculture, industrial applications, and the food industry [37, 39, 70]. Several routes of exposure are possible, although inhalation is considered the most likely route of exposure and also the most studied of all routes [24]. It is well established that GBNs are used in geoengineering to seed clouds [71, 72], which will pollute the air with GBNs. Also, the most concerning is the possible intranasal ingestion or insufflation of NPs through proposed aerosol spray vaccines, as well as experimental intranasal vaccination against influenza with a GBN complex [73]. Various commercial GBN-impregnated face masks were used in the global fight against COVID-19. Although the continuous wear of activated carbon masks has been established to pose a higher risk of fiber-like microplastic inhalation [74], the risk of inhaling GBNs from masks has not been researched extensively. However, inhaled GNSs can easily penetrate the tracheobronchial airways and then travel down to the lower lung airways, where it destroys the ultrastructure and biophysical properties of the pulmonary epithelial GL layer [27, 43], therefore, the first line of innate host defense [44]. GO was demonstrated to disrupt the alveolar-capillary barrier, allowing inflammatory cells to infiltrate the lungs, thus stimulating the release of pro-inflammatory cytokines, resulting in epithelioid granulomas, interstitial inflammation, and lung fibrosis [75–77]. GBNs caused inflammation and remained in the lung on day 90 after a single intratracheal instillation [78], and even translocated to the lung lymph nodes after inhalation [79].

Although GO derivatives had no, or rather finite, intestinal adsorption in adult mice after oral administration and were rapidly excreted [27, 80], low-dose GO caused serious damage to the gastrointestinal tract in the offspring after maternal mice drank a suspension of GO. A low-dose GO that does not agglomerate, could easily attach to the surface of the gastrointestinal epithelial cells and cause destruction through its abundant sharp edges [81]. Wu et al. found in a study that prolonged exposure to GO leads to significant primary (intestine) and secondary (neurons and reproductive) organ damage. GO induced the loss of intestinal villi and translocated into the intestinal walls, causing a hyper-permeable intestinal barrier [82]. Bantun reviewed the significant impact of ingesting GBMs on altering the composition, diversity, and function of the gut microbiome [19]. They found that it led to enteric disorders, with numerous pathological changes resulting in colitis, lysosomal dysfunction, inflammation, shortened colon, resorbed embryo, retardation in skeletal development, low fetus weight, fetal mortality, and inflammatory bowel disease. The increased use of GBMs in the food system and as fertilizers, as well as the proposed use in clothing and hygiene products, remains concerning.

Because nanomaterials offer a new range of unique properties that are commercially exploitable, engineered NPs development and production have increased very rapidly over the last few decades. Of note, most nanomaterial experts are from various engineering professions**,** and although much research in biomedicine has investigated the biocompatibility of these materials, it was mostly done from a drug development perspective, while not enough in-depth *in vivo* studies have been performed to ascertain the impact it has on human health at the cellular and molecular level.

Degradation of Graphene-based Nanomaterials

More research on the environmental fate and biodegradation of GBMs is needed, as their environmental and health impacts are still largely unknown. This lack of current understanding should motivate research into the breakdown of GBMs to address potential environmental toxicity and health hazards.

Humic acid or Shilajit is known to be excellent antidotes to GBNs [43], by mitigating its acute toxicity by regulating the translocation and metabolic fluxes of GBNs *in vivo* [83]. Humic acid was found to increase disordered structure and surface negative charges and reduce GBN aggregation [84, 85]. Through immune modulation and reduction of oxidative stress, humic acid also exhibits potent antiviral, antioxidant, and anticarcinogenic properties [86, 87]. Rozhina et al. found that the joint application of GO and kaolin nanoclay reduced the negative cytotoxic effects of graphene by almost 20% [88]. Although Bentonite nanoclay is also recommended as an antidote to graphene-induced cytotoxicity, Di Ianni et al. demonstrated *in vitro* that pristine Bentonite induced pro-inflammatory

responses in alveolar epithelial cells (A549) [89].

Several mechanisms exist by which biological systems degrade GBMs, particularly GO. Human eosinophils produce an enzyme known as eosinophil peroxidase (EPO) in the presence of low concentrations of hydrogen peroxide (H_2O_2) and sodium bromide (NaBr); EPO can degrade GO [90]. Another enzyme produced by neutrophils in the presence of low concentrations of H_2O_2 is myeloperoxidase (MPO), which can also degrade GO sheets. Given the roles of human eosinophils and neutrophils, the degradation of GO can be thought to be immune mediated. Still, much is unknown about GBNs in the human body. H_2O_2 is involved in various redox signal transduction pathways. It is known to exert DNA damage, while aggregated GO sheets did not degrade in the presence of H_2O_2. Kotchey et al. established that horseradish peroxidase can create holes in GO sheets [91], while nitric oxide (NO) can also degrade GO [92].

On the production side, functionalization of GO with the compounds coumarin and catechol increases the efficiency of biodegradation [93]. In the environmental context, GO can be degraded by light [94] and specific bacteria, such as *Labrys* sp. WJW [95], which are also capable of degrading $C_{60,}$ or Buckminsterfullerene [96]. Furthermore, GO can be degraded by adding $FeCl_3$, H_2O_2 and UV light after ultrasonification and acidification, which presents a promising means of removing GO from wastewater [97].

Conclusion

Graphite is a well-researched natural carbon allotrope; however, graphene, GO, and rGO are man-made materials with yet unknown effects on biological systems [28]. Until recently, the health effects associated with the use of GBNs have been studied *in vitro* at the cellular level and in short-term animal models, but the long-term systemic effects *in vivo* in humans are largely unknown, as well as the complex signaling pathways that regulate GBN toxicity [27].

Surprisingly, the commercialization of NP-based therapeutics is increasing considerably with a rise in the number of available products on the market, especially in the field of cancer therapy. The NPs include polymeric carriers, lipid-based vehicles, metallic NPs, and GBNs. Today, GBMs are produced on a large scale and have found niche applications in many biomedical technologies. In 2018 it was already predicted that the GBM market could reach millions of dollars by 2020, with concerns expressed regarding the release of NP and GBM wastes into the environment [29, 34, 43], with associated health risks [98].

However, less than 10% of these NP-based products are translated into clinical applications. To date, GBNs, as nano-adjuvants and for drug delivery, have mostly been used in preclinical research. The application of GBNs is at this stage a very promising, but clinically ineffective, experimental therapy, with a long way to go before translational research will be conducted. Therefore, even though it has been championed as the nanomaterial of the future, up to 2019, none of the GBN applications have been approved for clinical trials. Furthermore, various scientists have expressed concern about the lack of sufficient *in vivo* studies on the toxicology of NPs and GBMs that are used for biomedical applications [9, 29, 39]. Therefore, it would be important to critically evaluate the potential short- and long-term health risks and toxicity hazards of GBNs after acute, subacute, and chronic exposures and by using more long-term *in vivo* models (small and large animals). However, it is concerning that the application of advanced nanomaterials and GBNs in future diagnostics, vaccines, and antiviral therapies is given priority over current preparedness strategies in clinical settings against viruses.

With carbon-based graphene being hailed as a "wonder material" and the graphene industry booming, driven by several large initiatives, such as the NIH's BRAIN and the European Graphene Flagship, it must be seen to what extent GBNs deliver in nanomedicine the great promise so often espoused, with environmental risks and long-term health adverse effects still difficult to assess. Carbon nanotubes became the first GBN to be added to the Swedish non-profit organization SIN (Substitute It Now) list, which attracted attention to the future of sustainable nanotechnology [99]. The reason for inclusion in the SIN list was that GBNs were suspected of causing cancer, damaging fertility, and/or the unborn child, and show limited degradation in the environment.

Further use of GBMs requires careful investigation of the health and environmental risks, some of which have been included in this review. Precaution guides the adoption of new material technologies, balanced against any potential benefits.

CRediT Author Statement

Conceptualization, **H.N. du Preez** and **M. Halma**; writing-original draft preparation, **H.N. du Preez**; writing-review and editing, **H.N. du Preez** and **M. Halma**; **H.N. du Preez** created all the figures presented. The graphical abstract and Fig. 3 was created with BioRender.com. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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