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Screening of toxic potential of graphene family nanomaterials using *in vitro* and alternative *in vivo* toxicity testing systems

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Objectives The widely promising applications of graphene nanomaterials raise considerable concerns regarding their environmental and human health risk assessment. The aim of the current study was to evaluate the toxicity profiling of graphene family nanomaterials (GFNs) in alternative *in vitro* and *in vivo* toxicity testing models.

Methods The GFNs used in this study are graphene nanoplatelets ([GNPs]–pristine, carboxylate [COOH] and amide [NH2]) and graphene oxides (single layer [SLGO] and few layers [FLGO]). The human bronchial epithelial cells (Beas2B cells) as *in vitro* system and the nematode *Caenorhabditis elegans* as *in vivo* system were used to profile the toxicity response of GFNs. Cytotoxicity assays, colony formation assay for cellular toxicity and reproduction potentiality in *C. elegans* were used as end points to evaluate the GFNs' toxicity.

Results In general, GNPs exhibited higher toxicity than GOs in Beas2B cells, and among the GNPs the order of toxicity was pristine > NH2 > COOH. Although the order of toxicity of the GNPs was maintained in *C. elegans* reproductive toxicity, but GOs were found to be more toxic in the worms than GNPs. In both systems, SLGO exhibited profoundly greater dose dependency than FLGO. The possible reason of their differential toxicity lay in their distinctive physicochemical characteristics and agglomeration behavior in the exposure media.

Conclusions The present study revealed that the toxicity of GFNs is dependent on the graphene nanomaterial's physical forms, surface functionalizations, number of layers, dose, time of exposure and obviously, on the alternative model systems used for toxicity assessment.

Keywords Alternative toxicity testing, Human bronchial epithelial cells, *Caenorhabditis elegans*, Graphene family nanomaterials

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Introduction

Graphene is typically composed of sp2-hybridized carbon atoms packed densely in a honeycomb crystal lattice arranged in a two-dimensional structure, resulting in a large surface area on both sides of the planar axis. Graphene and related materials, including few-layer graphene, graphene nanosheets, graphene nanoplatelets (GNPs), ultrafine graphite, graphene oxide(GOs), and reduced GO etc., have been identified as graphene family nanomaterials (GFNs) [1-3]. The huge versatility of the GFNs

not only aiming for their potential future applications in the domain of electronics, photonics, composite materials, energy generation and storage, sensors and metrology, and biomedicine, specifically, biosensors, bioimaging and therapeutics, tissue engineering, and drug delivery etc., but also raised serious concerns about their environmental and human health impacts [1,3-5].

In recent years, several groups have devoted their studies to the elucidation of graphenes and related materials with *in vitro* and *in vivo*, but contradictory outcomes were reported. Therefore, at present, it is not possible to make any generalized conclusions about the biological interactions and toxic effects of GFNs. Moreover, GFNs are comprised of different forms of graphene nanomaterials that are endowed with different physicochemical characteristics; hence, not much has been understood about their toxicological profiles and more detailed studies are required [1,6]. In particular, toxicity data on GNPs which possess the potential to pose as nanohazards due to their specific platelet shape are still limited in comparison with related congeners [3,7]

The aim of our present study was to evaluate and compare the toxic potentiality of different forms of GFNs in *in vitro* and alternative *in vivo* systems. We used five different commercially available forms of GFNs that consist of GNPs (with or without functionalization–pristine, carboxylate [COOH] and amide [NH_2]) and GOs (single layer [SLGO] and few layers [FLGO]). The human bronchial epithelial cells (Beas2B cells) and the nematode *Caenorhabditis elegans* were used as alternative *in vitro* and *in vivo* model systems, respectively, to profile the toxic responses of the GFNs.

Materials and Methods

Graphene Family Nanomaterials

The powdered GOs (SLGO and FLGO) and GNPs with different surface functionalization (pristine, COOH and NH_2) were purchased from Cheap Tubes.com (http://www.cheaptubes.com/) and stocks prepared in distilled water at 1000 mg/L with sonication.

Cell Culture and Graphene Family Nanomaterials Treatment

Beas2B cells were cultured in Dulbecco's modified Eagle medium/F12(DMEM/F12; GIBCO Life Science, Great Island, NY, USA), supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics, at 37°C in a 5% CO_2 atmosphere.

The GFNs were freshly prepared in cell culture medium DMEM/F12 at the desired concentrations with appropriate amounts of each stock (1000 mg/L in distilled water) and sonicated for 10 minutes just before biological exposure.

Cytotoxicity and Cell Viability Assessment

The cytotoxicities of all GFNs were determined with the EZ-Cytox cell viability assay kit (Daeil Lab Service, Seoul, Korea) based on cleavage of the tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase, as described previously [8]. Approximately, 5×10^3 cells/well were seeded in 96-well plates 24 hours prior to treatment and exposed to a range of concentrations (5 to 150 mg/L) of five different GFNs for the next 24 hours. Next, in a separate experiment, the cells (in 96-well plates) were exposed to all GNPs (20 mg/L) and GOs (50 mg/L) for different time point (4 to 72 hours). After completion of the exposure time, 10 μL of EZ-Cytox reagent was added to each well including treated and control (without GFNs). Absorbance was detected at 450 nm after 2 hours of incubation at 3°C. Appropriate blanks were used for each concentration to validate the absorbance.

In addition, the cells (5×10^4 cells/well in a 6-well plate) were also exposed to a fixed concentration (20 mg/L) of GFNs in complete and serum free DMEM/F12 medium for 24 hours. The cell viability was measured using the standard trypan blue (Invitrogen, Carlsband, CA, USA) staining method and the total numbers of stained and unstained cells counted using a hemocytometer.

Colony Formation and Morphology Changes

The colony formation assays were carried out as described by Herzog et al. [9]. Exponentially growing cells were harvested and seeded in six-well culture plates at a density of 250 cells/well. Each well contained 2 mL of cell culture medium. Cells were allowed to attach for approximately 24 hours. The cells were then washed with 2 mL of phosphate buffered saline (PBS) and treated with 2 mL of nanoparticles prepared in cell culture medium to final concentrations of 10 and 50 mg/L. Cells were exposed to nanoparticles over the time period they needed to form colonies, a colony being defined as at least 50 clones of one cell and incubated for 10 days. Before colonies were counted, the particle solutions were removed, the cells were washed with PBS and finally fixed and stained using a 0.1% Giemsa (Sigma-Aldrich, St Louis, MO, USA). Colony shape and cell morphology were detected under a light microscope.

Maintenance of *C. elegans*

C. elegans were grown in Petri dishes on nematode growth medium and fed with *Escherichia coli* strain OP50 according to a standard protocol [10]. The worms were incubated at 20°C and

young adults (3 days old) from age-synchronized cultures were used in all the experiments.

Reproduction (72 Hours Assays) of *C. elegans*

The effects of GFNs on the reproduction of wild type worms were investigated as described by Roh et al. [11]. After a young adult was exposed to GFNs at various concentrations (5 to 50 mg/L mixed in K-media) for 72 hours, the number of offspring at all stages beyond the egg were counted. Five replicates were conducted for reproduction assays.

Statistical Analysis

The statistical significance of differences among/between treatments was determined using one way analysis of variance. This was followed by a post-hoc test (Tukey, $p < 0.05$). All statistical analyses were carried out using SPSS version 12.0 KO (SPSS Inc., Chicago, IL, USA) and graphs were prepared in SigmaPlot version 12.0 (Systat Software, Inc., Chicago, IL, USA).

Results

Graphene Family Nanomaterials Characterization

The details of layer number, thickness, dimensions, functionalization, methods of preparation etc. supplied by the manufacturer are summarized in Table 1. We used pristine and two other functionalized (COOH and NH_2) GNPs of similar layer numbers, lateral dimensions, and average thickness, and two kinds of GOs which only differed in layer number - SLGO and FLGO (4 to 8 layer) but were of similar dimensional sizes.

Effects on Cell Viability and Cytotoxicity

At first the viabilities of Beas2B cells were evaluated at various concentrations of GFNs for 24 hours. In general, a dose dependent decrease in viability was not markedly clear at lower doses (5 to 25 mg/L) but dose dependency became much more profound at higher doses (25 to 150 mg/L, Fig 1A). Unlikely, the GFNs induced decrease in viability at a dose of 20 mg/L was found to be clearly time dependent and become more distinct after 16 hours of exposure (Figure 1B). Furthermore, no significant difference in viability was observed between complete media and serum free media at a dose of 20 mg/L (Figure 1C). In general, GNPs were found to be more toxic than GOs. The order of sensitivity of Beas2B cells towards the GNPs were found as pristine > NH_2 > COOH. The COOH and NH_2 functionalization showed more or less similar cytotoxic effects on Beas2B cells, which only became different at the highest dose (150 mg/L) and longest time (72 hours) point (Figure 1A, 1B). Interestingly, GOs mediated decreases in viabilities were not as profound as those induced by GNPs and both compounds showed more or less similar cytotoxicity, except at the highest dose (150 mg/L) and longest time (72 hours) point where SLGO become more toxic than FLGO (Figure 1A, 1B).

Colony Formation and Morphology Changes

Significant dose dependent decreases in colony numbers were evident due to 10 days of exposure of GFNs (Figure 2A, 2B)

Table 1. Characterization of graphene nanomaterials (as supplied by the manufacturer)

Properties	GOs		
	Single-layered		Few-layered
Thickness	0.7-1.2 nm		4-8 layers
X &Y dimensions (nm)	300-800		300-800
No. of layers	1		4-8
Purity (wt%)	>99.0		>99.0
Method	Modified hummers		Modified hummers
Solubility	Water, NMP, DCB, DMF		Water, NMP, DCB, DMF
	GNPs		
	GNP-pristine	GNP-COOH	GNP-NH_2
Lateral dimensions (μm)	1-2	1-2	1-2
Average thickness (nm)	<4	<3	<3
No. of layers	<4	<3	<3
Purity (wt%)	>99	>99	>99
Surface area (m^2/g)	>750	>750	>750
Plasma process gas	Argon	Proprietary oxygen based	Nitrogen
Primary functionality	None	COOH	NH_2
Other functionality	Atmospheric gas	COH, C=O, other oxygen	N-H, O=C-N-H_2, C=N
Source material	Natural graphite	Natural graphite	Natural graphite
Form supplied	Dry powder	Dry powder	Dry powder

GOs, graphene oxides; NMP, n-methyl-2-pyrrolidone; DCB, dichlorobenzene; DMF, dimethylformamide; GNPs, graphene nanoplatelets.

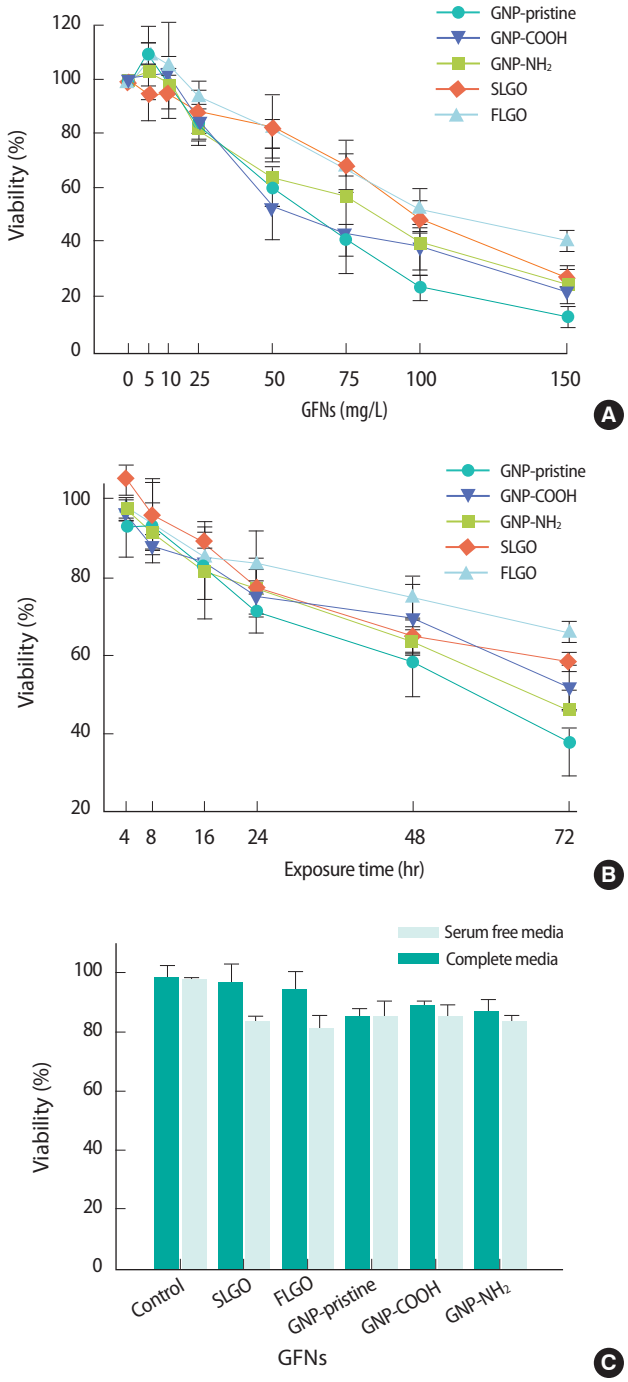

Figure 1. Cytotoxicity of GFNs to Beas2B cells. (A) The viability (%) determined by EZ-Cytox assay after 24 hours of exposure to GFNs in complete DMEM/F12 medium. (B) Cells exposed to GNPs (at 20 mg/L) and GOs (at 50 mg/L) at different time points and cytotoxicity determined by EZ-Cytox assay. (C) The viability (%) determined by trypan blue exclusion method after 24 hours of exposure to GNPs (at 20 mg/L) and GOs (at 50 mg/L) in complete DMEM/F12 media and serum free media. Data are presented as mean±standard error of mean. GFNs, graphene family nanomaterials; Beas2B, human bronchial epithelial; DMEM/F12, Dulbecco's modified Eagle medium/F12; GNPs, graphene nanoplatelets; GOs, graphene oxides; SLGO, single layer GO; FLGO, few layers GO; COOH, carboxylate; NH_2, amide.

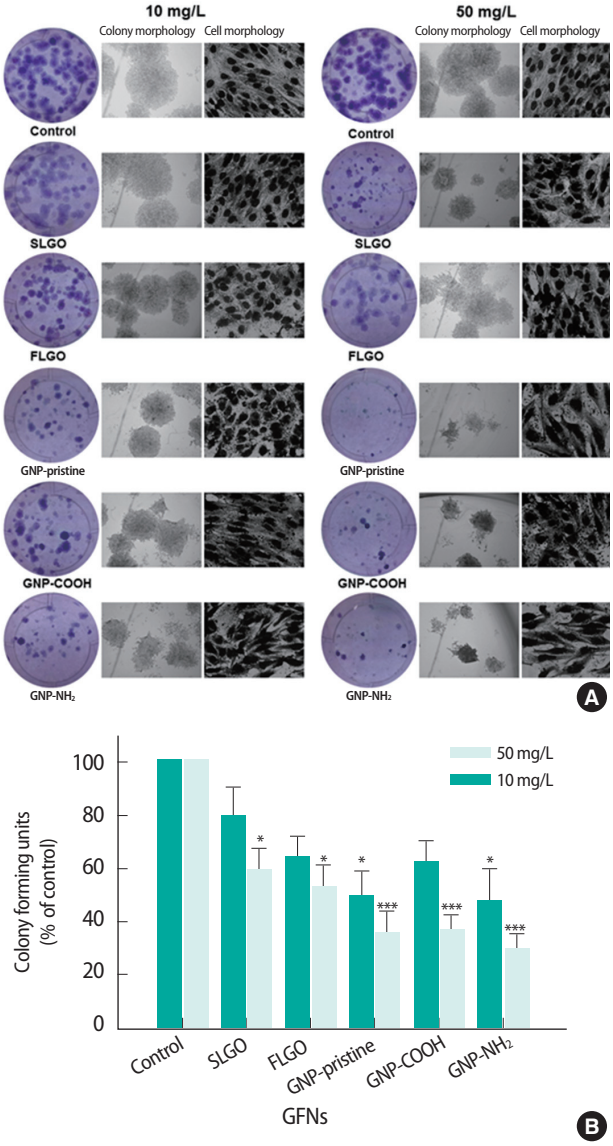

Figure 2. The effects of GFNs exposure on colony formation in Beas2B cells for 10 days. (A) Colonies and cell morphology of Beas2B cells after 10-day exposure to GFNs at doses of 10 and 50 mg/L. (B) Effects on colony number. Data are presented as percent of control mean±standard error of mean. GFNs, graphene family nanomaterials; Beas2B, human bronchial epithelial; SLGO, single layer graphene oxide; FLGO, few layers graphene oxide; GNPs, graphene nanoplatelets; COOH, carboxylate; NH_2, amide. *p<0.05, ***p<0.001.

with respect to control cells (without GFNs). Likewise the acute cytotoxic effects, GNPs exposure caused much higher effects on colony numbers and fragmentation than GOs. The dose dependency was much sharper in SLGO, that is to say, at a lower dose (10 mg/L) both SLGO and FLGO showed similar effects on colony formation, but SLGO affected Beas2B cell colonies at a higher dose (50 mg/L) more profoundly than FLGO. The potency of the effects on colony formation of the GNPs was COOH > NH_2 > pristine. In addition, a significant deforma-

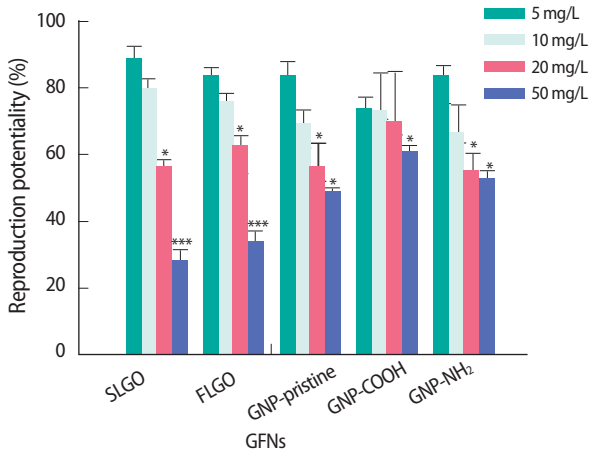

Figure 3. Effect of GFNs treatment on *C. elegans*. Percentage of reproductive potentiality of wild-type *C. elegans* due to GFNs exposure at different doses (5, 10, 20, and 50 mg/L) for 72 hours. Data are presented as mean±standard error of mean. GFNs, graphene family nananomaterials; SLGO, single layer graphene oxide; FLGO, few layers graphene oxide; GNPs, graphene nanoplatelets; COOH, carboxylate; NH_2, amide. *p<0.05, ***p<0.001.

tion in terms of morphological characteristics was also observed at both doses (10 and 50 mg/L) of GNPs and at the higher (50 mg/L) dose of SLGO (Figure 2A) on Beas2B cell colonies.

Effects of Graphene Family Nananomaterials on Reproduction of *C. elegans*

Clear and marked dose dependent decreases, except with GNP-COOH, on reproductive potentiality were evident in GFNs' treated *C. elegans* (Figure 3). Unlike *in vitro* effects, GNPs did not show greater reproductive toxicity than GOs. Conversely, GOs were evident as the most reproductively toxic compounds among all the GFNs. The GNPs showed almost the same level of toxicities in *C. elegans* irrespective of functionalization effects at the lowest dose of 5 mg/L. However, dose dependency became clear in GNP-pristine and GNP-NH_2 with increasing doses. Interestingly, no dose dependency was observed in GNP-COOH exposed worms, except a moderately significant decrease at the highest dose (50 mg/L, $p < 0.05$).

Discussion

In the current study we evaluated the *in vitro* toxic potentialities of GFNs in Beas2B cells and the influence of surface functionalization (pristine, NH_2 and COOH) and layer number dependency (SLGO and FLGO) on their biological interaction. Moreover, we used the nematode *C. elegans* as an alternative *in vivo* model system to achieve better identification of GFNs mediated environmental health hazards.

Effect of Different Types of Graphene Family Nanomaterials

The biological effects of multiple graphene forms must be taken into account because the different types possess unique physicochemical properties and, hence, exert different biological/toxicological phenomena [6]. The GNPs possessed greater cellular toxicity than GOs due to the physical interaction and the cell membrane damage by the hydrophobic agglomerates formed, as was found with reduced GO [12]. Moreover, their platelet like structural shape possibly is the basis for the higher toxicity of GNPs in Beas2B cells (Figures 1A and 2B) compared to GOs [7]. In contrast, hydrophobic agglomerates of GNPs in aqueous solution hinder their biocompatibility and availability to worms and thus become less toxic in *C. elegans* (Figure 3) with respect to reproductive capability [13].

Surface Functionalization Effect

The oxidative functionalization in GOs makes them hydrophilic and much more biocompatible than GNPs. The main reason for the differential mode of toxicity of GFNs is their hydrophilicity/hydrophobicity and dispersion in the exposure medium, i.e., whether or not aggregation occurs [12,14]. Furthermore, among the GNPs, pristine was found to be the most toxic form (Figures 1-3) in both model systems – Beas2B cells as well as *C. elegans*. Possibly GNP-pristine led to greater agglomeration and cell membrane damage than GOs, as was previously reported for graphene-pristine in comparison with graphene-COOH [15]. Likewise multi-walled carbon nanotube-pristine as was documented by Chatterjee et al. [16], the possible accumulation of GNP-pristine in reproductive organs caused a reduction in the reproductive potentialities in worms. Covalent functionalization (such as -COOH) can decrease the toxic potentialities of GNPs by enhancing their hydrophilicity and clearance, as was suggested for carbon nanotubes [3,17].

Layer Number Dependency

The SLGO and FLGO possess similar lateral dimensions with different numbers of layers. Although similar trends in toxicity were observed in both SLGO and FLGO, clearer and more specific dose dependency was evident in SLGO in both model systems with all tested end points. It is expected that the biological interactions of SLGO would be greater than those of FLGO as their stiffness, a characteristic which is inversely proportional to biological adsorptive capability, increases in FLGO with an increase in the number of layers [3,18].

Model System Specificity

In general, GNPs (pristine, COOH, and NH_2) were found to

be more toxic in Beas2B cells and GOs (SLGO and FLGO) were more toxic to the nematode *C. elegans'* reproduction capability. Although it seems contradictory, but the probable answer lays in their physicochemical characteristics, in particular the extent of agglomeration formation in the exposure medium, which in turn governs their biological interactions. The GNPs aggregation behavior possibly helps the worms to avoid ingesting them but, if ingested, they then persist for a longer time and possibly accumulate in the reproductive system, causing reproductive failure. Conversely, the nature of dispersion of GOs caused higher exposure (through ingestion as well as other absorption routes) in the worms, making them more vulnerable at higher treated doses. In the case of cellular toxicity, the Beas2B cells adhered in monolayers and possibly were subjected to sharp physical interactions and cell membrane damage from GNPs' agglomerates, while dispersed GOs were taken up by cells and further biological interactions occur. A similar kind of differential observation between suspended cells and adherent cells were reported that the GOs showed the greater hemolytic activity in suspended red blood cells than aggregated graphene sheets, whereas graphene sheets exhibited a greater capacity to damage mammalian fibroblasts (adherent cells) [14]. Taken together, clearly distinguishable toxic responses of GFNs were observed between *in vitro* (Beas2B cells) and *in vivo* (the nematode *C. elegans*).

Our study revealed that the toxicity of graphene is dependent on the graphene nano type, surface functionalization, number of layers, dose, and time of exposure. Moreover the in vitro-in vivo-differential toxicity should be taken into specific consideration. Therefore, the generalization of the toxicity profiles of GFNs must be avoided. We believe that the data presented in this paper would have the potentiality to be used in the field of occupational and human health risk assessment as well as the biomedical application domain. This study also suggests the potential of using alternative toxicity systems, for instance, the nematode *C. elegans*, in screening the toxicity of new chemicals, such as, nanomaterials.

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Conflict of interest

The authors have no conflicts of interest with material presented in this paper.

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