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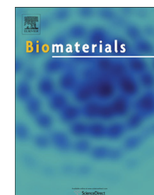

# A systems toxicology approach to the surface functionality control of graphene–cell interactions

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ABSTRACT

The raised considerable concerns about the possible environmental health and safety impacts of graphene nanomaterials and their derivatives originated from their potential widespread applications. We performed a comprehensive study about biological interaction of grapheme nanomaterials, specifically in regard to its differential surface functionalization (oxidation status), by using OMICS in graphene oxide (GO) and reduced graphene oxide (rGO) treated HepG2 cells. Differential surface chemistry (particularly, oxidation – O/C ratio) modulates hydrophobicity/philicity of GO/rGO which in turn governs their biological interaction potentiality. Similar toxic responses (cytotoxicity, DNA damage, oxidative stress) with differential dose dependency were observed for both GO and rGO but they exhibited distinct mechanism, such as, hydrophilic GO showed cellular uptake, NADPH oxidase dependent ROS formation, high deregulation of antioxidant/DNA repair/apoptosis related genes, conversely, hydrophobic rGO was found to mostly adsorbed at cell surface without internalization, ROS generation by physical interaction, poor gene regulation etc. Global gene expression and pathway analysis displayed that TGFβ1 mediated signaling played the central role in GO induced biological/toxicological effect whereas rGO might elicited host-pathogen (viral) interaction and innate immune response through TLR4–NFkB pathway. In brief, the distinct biological and molecular mechanisms of GO/rGO were attributed to their differential surface oxidation status.

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## 1. Introduction

Graphene is a single-atom-thick sheet of sp2-bonded carbon atoms in a closely packed honeycomb two-dimensional lattice structure, isolated from its three-dimensional parent material, graphite. The members of graphene nanomaterials include few-layer-graphene (FLG), ultrathin graphite, graphene oxide (GO) (monolayer to few layers), reduced graphene oxide (rGO), and graphene nanosheets (GNS). As the names suggest, graphene oxide (GO) is highly oxidized form of chemically modified graphene and the reduced graphene oxide (rGO) is the products of treating GO under reducing conditions, which include thermal/chemical treatments with reducing agents. The conversion to rGO alters many properties of GO, such as, electrical conductivity, increases hydrophobicity, reduces surface charge, water dispersibility etc. [1]. The unique physicochemical properties of graphene (for e.g., large surface area, extraordinary electrical and thermal conductivities, strong mechanical strength, capability of bio-functionalization and mass production) have drawn lot of research interests toward exceptional promise of applications, for instances, biosensing/bioimaging (optical sensing, fluorescence imaging probes, electrochemical sensing), electronic devices (transistors, solar cells, matrix for mass spectra), disease diagnosis, gene and drug delivery, cancer therapy, photothermal therapy bacterial inhibition, antibacterial papers, antiviral materials, tissue engineering etc. [1–3].

In lieu of the above mentioned growing research interests in potential application of graphene nanomaterials (GNMs) simultaneously evoke the concern about their possible environmental health and safety (EHS) impact from its wide use. To this end the several studies reported in vitro toxicity of GNMs in bacterial [4,5], adherent mammalian [3,6,7], cancerous cells [8] or suspended cells [9] and in comparison only few studies were documented with in vivo toxicity of GNMs in *Caenorhabditis elegans* [10] and mouse [11]. Toxicity of graphene family materials was compiled and reviewed by Jastrzebska et al. recently [12]. Most studies were found to focus on pristine graphene, sheets/platelets of graphene nanomaterials, graphene oxide (GO) whereas only few studies were available for reduced graphene oxide (rGO). The underlying mechanism of toxicity of GNMs was reported as oxidative stress

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[1,4,5], genotoxicity [3], apoptosis [6], autophagy [13], immune responses [14] etc. which extensively varied with the particular physiocochemical properties of GNMs, such as surface area, layer number, lateral dimension, surface chemistry, purity etc. The biological interactions of graphene family nanomaterials, specifically in regard of their physiocochemical properties, were discussed by Sanchez et al. in their recent review [1].

Previous studies revealed the complex interactions between graphene nanomaterials and biological systems mainly probed the cytotoxicity, uptake, genotoxicity, oxidative stress etc. but no studies were carried out with system biology approach to reveal comprehensive genomic regulation and molecular mechanism exerted by GNMs. The mechanism based predictive toxicology approach was suggested as necessary approach to explain the various types of biophysicochemical interactions at the nano–bio interface [15]. To the best of our knowledge, none of the work has addressed this issue in graphene nanomaterials with system biology and OMICS approach. Moreover, no studies have documented the comparative toxicity effect of GO and rGO liver. In the present study, two commonly used derivatives of GNMs, graphene oxide (GO) and reduced graphene oxide (rGO), were selected as the testing nanomaterials and human hepatoma HepG2 cells were chosen as the in vitro model for studying the potential liver toxicity. Herein, we addressed mainly two questions: i) How the altered surface chemistry (oxidation) of graphene nanomaterials (GO/rGO) influences cellular interaction? ii) Is it markedly different, if so, what is the molecular mechanism for their distinct cellular functions? For this purpose, the physicochemical properties of GO/rGO were extensively characterized with atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), Raman spectroscopy, Transmission electron microscopy (TEM), dispersion ability etc. and the biological properties were conducted in GO/rGO treated HepG2 cells to evaluate uptake, cytotoxicity, oxidative stress–antioxidant gene expression, DNA damage–repair gene expression, mode of cell death (apoptosis/necrosis) and most importantly global gene expression and pathway analysis. In addition, GO exposed mouse's whole blood and liver tissues were also used for DNA damage and gene expressions, respectively.

## 2. Materials and methods

### *2.1. Graphene nanomaterial characterizations*

The commercial graphene nanomaterials, graphene oxide (GO) and reduced graphene oxide (rGO), were purchased from the UniNanoTech (UniNanoTech Co., Yong-In, Korea) and were characterized by using atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), transmission electron microscopy (TEM) and Raman spectroscopy. Surface topography, height profile and lateral size distribution of the GO, RGO were examined by AFM (Park Systems XE-BiO) in non-contact mode. The samples for the AFM imaging were prepared by drop casting a diluted suspension (GO-300 mg/L, RGO-275 mg/L) onto a cleaned mica substrate and dried at room temperature for 24 h. XPS was used to examine the surface chemical states of the GO and the RGO. The data were obtained by a hemispherical analyzer equipped by a monochromated Al X-ray sources (Al K$\alpha$ line: 1486.6 eV) operating at a vacuum ($4.6 \times 10^{-9}$ mb). The XPS peaks were analyzed by using Gaussian components after a Shirley background subtraction. The O/C atomic ratios of the samples were obtained by using peak area ratios of the XPS core levels and the sensitivity factor (SF) of each element in XPS. Raman spectroscopy was performed at room temperature with a Micro Raman system (UniRAM3500, UniNanoTech Co., Ltd., Korea) with a 532 nm laser. The calibration was initially made using an internal silicon reference at 500 $cm^{-1}$ and gave a peak-position resolution of less than 1 $cm^{-1}$. The spectra were measured from 500 to 3000 $cm^{-1}$. For XPS and Raman spectroscopy powdered samples of GO/rGO were used. Structure and layer number were investigated by TEM (Carl Zeiss LIBRA 120). The samples for TEM were prepared by drop casting a diluted suspension (50 mg/L) onto a carbon film with 300 square mesh copper grids and dried at room temperature for 24 h. The size distribution and $\zeta$-potential of the GO/rGO (10 mg/L in MEM culture media) were evaluated by using a Photal dynamic light scattering spectrometer (DLS) (DLS-7000, Otsuka Electronics Co., Inc.).

### *2.2. Cell culture and GO/rGO treatment*

HepG2 (human liver carcinoma cells) were cultured in MEM (GIBCO), supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics, at 37 °C in a 5% $CO_2$ atmosphere.

Commercial GO was directly used as a stock (275 mg/L) and rGO stock was prepared in distilled water (300 mg/L) and before biological exposure, the rGO suspension was sonicated for 5 min. The appropriate amounts of GO/rGO were dispersed in the cell culture medium (MEM) to achieve the desired concentrations for respective experiments.

### *2.3. Mouse maintenance and experiment*

The details are described in Supplementary materials (Material method section).

### *2.4. Cytotoxicity and cell viability*

Cytotoxicity of GO/rGO was determined by EZ-Cytox cell viability assay kit (Daeil Lab Service, Korea) based on the cleavage of the tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase. Approximately, $5 \times 10^3$ cells/well were seeded in 96-well plates 24 h prior to treatment and exposed to a range of concentrations (from 1 mg/L to 200 mg/L) of GO/rGO for another 24 h. In addition, the cells (in 96-well plates) were exposed to GO/rGO (respective EC20 dose) for different time point (from 4 h to 72 h). After that10 μL of EZ-Cytox reagent was added to each well including treated and control (without GO/rGO). Absorbance ($OD_{450}$) was detected at 450 nm after 3 h of incubation at 37 °C. Appropriate blanks were used for each concentration to validate the absorbance. $EC_{50}$s were calculated by using 4 parametric logistic equations.

The cell viability was measured using standard trypan blue (Invitrogen) staining method and the total numbers of stained and unstained cells counted using a hemocytometer.

### *2.5. GO/rGO uptake*

#### *2.5.1. Uptake detection by flowcytometry and image analysis*

Cells were grown in 6-well plates at $5 \times 10^4$ cells/ml and then treated with 20 mg/L of GO/rGO for 24 h. After treatment the cells were harvested, washed thrice in cold PBS and analyzed by flowcytometry. For imaging of uptake, cells ($5 \times 10^4$ cells/ml) were plated onto glass coverslips in 6-well plates and treated with GO/rGO (their respective EC20 and EC50 doses) for 24 h at 37 °C. Cells were washed thrice in cold PBS and the images (Leica DCF 290C) were acquired under DIC microscope (Leica DM2500) at 100× magnification.

#### *2.5.2. Blocking uptake using inhibitors of endocytosis*

Approximately, $5 \times 10^3$ cells/well were seeded in 96-well plates 24 h prior to treatment. Then the cells were preincubated with various inhibitors: methyl-β-cyclodextrin (2.5 mg/mL), LY294002 (20 μg/mL), chlorpromazine (1 μg/mL) and sodium azide (6.5 μg/mL) for 1 h followed by addition of GO/rGO (respective EC50 doses) and after incubation of 24 h, cytotoxicity was measured with EZ-Cytox method as described previously. Dose of the inhibitors were selected as described by Fernando et al. [16] and were adjusted by testing less than 10% cytotoxicity with the inhibitor alone (data not shown).

### *2.6. Oxidative stress measurement*

#### *2.6.1. Intracellular ROS measurement*

Intracellular ROS production was monitored by using 2,7-dichlorodihydrofluorescein diacetate (DCFDA) (Molecule Probes Inc., Eugene). HepG2 cells ($5 \times 10^4$ cells/ml) were plated onto glass coverslips in 6-well plates and treated with GO/rGO (their respective EC20 and EC50 doses) for 0.5 h, 1.5 h, 4 h and 24 h at 37 °C. After respective time point incubation cells were stained with DCFDA (25 μM) for 30 min at 37 °C in dark and fluorescence measured under microscope (Leica DM IL).

#### *2.6.2. GSH, GSSG and malonaldehyde (MDA) measurement*

HepG2 cells ($5 \times 10^4$ cells/ml) were plated onto 100 mm plates and treated with GO/rGO (their respective EC20 and EC50 doses) for 24 h at 37 °C. Cells were harvested, washed with PBS, pelleted and crude cell extract prepared in 50 mM phosphate buffer and used for GSH, GSSG and MDA measurement. Intracellular reduced (GSH) and oxidized (GSSG) glutathione levels were measured by EnzyChrom™ GSH/GSSG kit (EGTT-100, Bioassay Systems) and presented as GSH/GSSG ratio. Lipid peroxidation was determined by measuring the MDA level by standard thiobarbituric acid (TBA). Each experiment was carried out with three replicates.

#### *2.6.3. Blocking ROS formation using N-acetylcystine (NAC), trolox and diphenylene iodonium*

Approximately, $5 \times 10^3$ cells/well were preincubated in 96-well plates 24 h prior to treatment. Then the cells were preincubated with NAC (10 μg/mL), trolox (10 μg/mL) and diphenylene iodonium (DPI) (50 nM, the inhibitors of NADPH oxidase) for 1 h followed by addition of GO/rGO (respective EC50 doses) and after incubation of 24 h, cytotoxicity was measured with EZ-Cytox method as described previously. Dose of the DPI was selected by testing less than 10% cytotoxicity with the DPI alone (data

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