Ecotoxicology and Environmental Safety 159 (2018) 221–231

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Characterization of the effects of trace concentrations of graphene oxide on zebrafish larvae through proteomic and standard methods

Wei Zou[a], Qixing Zhou[a], Xingli Zhang[a], Li Mu[b,*], Xiangang Hu[a,*]

[a] Key Laboratory of Pollution Processes and Environmental Criteria (Ministry of Education)/Tianjin Key Laboratory of Environmental Remediation and Pollution Control, College of Environmental Science and Engineering, Nankai University, Tianjin 300071, China

[b] Tianjin Key Laboratory of Agro-environment and Safe-product, Key Laboratory for Environmental Factors Control of Agro-product Quality Safety (Ministry of Agriculture), Institute of Agro-environmental Protection, Ministry of Agriculture, Tianjin 300191, China

ARTICLE INFO

Keywords:
Graphene oxide
Proteome
Mitochondrial damage
Oxidative stress
Cardiac toxicity

ABSTRACT

The effects of graphene oxide (GO) carbon nanomaterials on ecosystems have been well characterized, but the toxicity of GO at predicted environmental concentrations to living organisms at the protein level remain largely unknown. In the present work, the adverse effects and mechanisms of GO at predicted environmental concentrations were evaluated by integrating proteomics and standard analyses for the first time. The abundances of 243 proteins, including proteins involved in endocytosis (e.g., *cltcb*, *arf6*, *capzb* and *dnm1a*), oxidative stress (e.g., *gpx4b*, *sod2*, and *prdx1*), cytoskeleton assembly (e.g., *krt8*, *krt94*, *lmna* and *vim*), mitochondrial function (e.g., *ndufa10, ndufa8, cox5aa,* and *cox6b1*), Ca^{2+} handling (e.g., *atp1b2a*, *atp1b1a*, *atp6v0a1b* and *ncx4a*) and cardiac function (e.g., *tpm4a*, *tpm2*, *tnni2a.1* and *tnnt3b*), were found to be notably altered in response to exposure 100 μg/L GO. The results revealed that GO caused malformation and mortality, likely through the downregulation of proteins related to actin filaments and formation of the cytoskeleton, and induced oxidative stress and mitochondrial disorders by altering the levels of antioxidant enzymes and proteins associated with the mitochondrial membrane respiratory chain. Exposure to GO also increased the heart rate of zebrafish larvae and induced pericardial edema, likely by changing the expression of proteins related to Ca^{2+} balance and cardiac function. This study provides new proteomic-level insights into GO toxicity against aquatic organisms, which will greatly benefit our understanding of the bio-safety of GO and its toxicity at predicted environmental concentrations.

1. Introduction

Due to their excellent optical, mechanical, electronic, and catalytic properties, graphene-family nanomaterials (GFNs) have attracted great attention in various fields. Graphene oxide (GO) has been widely applied in the biomedical and environmental fields, such as in sensors (Kim et al., 2017; Tiwari et al., 2016), drug delivery (Dowaidar et al., 2017), membranes for desalination, separation and water purification (Finnerty et al., 2017; Abraham et al., 2017; Williams and Carbone, 2016; Liu et al., 2017), and adsorbents for wastewater or drinking water treatments (Khan et al., 2017; Zambianchi et al., 2017). The annual production of GFNs was estimated to have equaled 120 metric tons in 2015 and is expected to reach 1200 metric tons by 2019 (Zurutuza and Marinelli, 2014), and the market for GO is projected to reach nearly $675 million by 2020 (Ahmed and Rodrigues, 2013). Due to increases in their production and application, GO materials will likely be released into the environment during their lifecycle (Goodwin et al., 2018; Du et al., 2017; Wang et al., 2017; Suárez-Iglesias et al., 2016). For example, GO-polymer nanocomposites could release GO particles when exposed to UV radiation (Bernard et al., 2011). An analysis of the lifecycles of nanomaterials indicated that the fate of GO is likely to be similar to that of carbon nanotubes: more than 80% of manufactured GO will potentially be released into the environment (Lanphere et al., 2014). For example, GO might be released into water environments during environmental applications, such as from adsorbents for wastewater and drinking water treatment, membranes for desalination, and catalysts for the degradation of aqueous organic pollutants (Zhao et al., 2014; Goodwin et al., 2018).

The increasing production of GFNs has raised concerns regarding their potential health and ecological risks (Suárez-Iglesias et al., 2016). Studies have found that GO can translocate into target organs and interact with tissues, cells, organelles and other biological macromolecules, resulting in abnormal functions in organisms (Hu et al., 2014; Hu and Zhou, 2013; Ouyang et al., 2015). For example, GO

* Corresponding authors.
E-mail addresses: muli666up@163.com (L. Mu), huxiangang@nankai.edu.cn (X. Hu).

https://doi.org/10.1016/j.ecoenv.2018.05.009
Received 27 December 2017; Received in revised form 1 May 2018; Accepted 3 May 2018
Available online 21 May 2018
0147-6513/

induces oxidative stress, cytotoxicity, pulmonary injury, genotoxicity, and reproductive toxicity in cells and organisms (Souza et al., 2018; El-Yamanya et al., 2017; Ema et al., 2016). The results from studies conducted with high doses of GO (e.g., parts per million or higher) are difficult to extrapolate to humans or organisms in real contaminated environments (Hu et al., 2016). However, few studies have been conducted using predicted environmental concentrations of GO, such as at parts per billion (μg/L) or parts per trillion (ng/L) (Zhang et al., 2017). In addition, most studies have been limited to the analysis of standard parameters using cytological, biochemical and histological methods. Very few studies have used omics techniques to obtain a more in-depth understanding of the mechanisms of GO nanotoxicity. Zebrafish constitute a well-accepted model for the testing of nanomaterial toxicity, and the results obtained with zebrafish are relevant to human health (Paatero et al., 2017; Jeong et al., 2015). Based on the abovementioned assumption, information on the toxicity of GO at predicted environmental concentrations to zebrafish is critical for assessing the potential ecological and health risks of GO. Our studies and those of other researchers have found that GO can translocate from water to zebrafish, and this translocated GO affects zebrafish embryonic development by producing ROS, damages mitochondria, and induces genotoxicity and metabolite dysfunction (Souza et al., 2017; Chen et al., 2016; Ren et al., 2016). However, the proteome- and molecule-level mechanisms underlying the aquatic toxicity of GO have remained obscure until now. Proteomics is an important tool for the assessment of nanomaterial risks (Matysiak et al., 2016). Changes in protein expression directly reflect the changes in cellular biological processes in response to an external stimulus, which allows the prediction and identification of an organism's response to that stimulus (Kjeldal et al., 2016). Proteomics has been used to evaluate the toxicity of cadmium sulfide quantum dots (CdS QDs), cerium oxide nanoparticles (CeO_2 NPs) and gold nanoparticles (AuNPs) (Pasquali et al., 2017; Salehi et al., 2017; Ng et al., 2015) at ppm levels.

In the present study, proteomic profiling was performed using liquid chromatography-tandem mass spectrometry (LC−MS/MS) to simultaneously detect and quantify the protein expression profiles of zebrafish larvae and larvae exposed to GO at predicted environmental concentrations (μg/L). Standard analyses, including assays of mortality, the mitochondrial transmembrane potential, Na^+/K^+-ATPase activity and the glutathione content, were conducted to further confirm and support the proteomic predictions. This work will provide useful information for understanding the toxicity of GO at the proteomic level.

2. Materials and methods

2.1. GO characterizations

GO with a purity greater than 99% was purchased from XFNANO (XF002-1, Nanjing, China) and was synthesized using Hummers' method. The characteristics of GO were described in detail previously (Zhang et al., 2017). Briefly, GO exhibits a typical nanosheet morphology with a thickness of 1.01 ± 0.05 nm and a lateral length ranging from approximately 0.3 to 2.6 μm. GO is composed of 64.6% C1s, 31.4% O1s, 1.9% N1s and 2.1% S2p. Specifically, C1s includes 4.1% C-C=O, 50.6% C- C, 20.0% C-O and 25.3% C=O, and the O1s peak includes 75.3% C=O and 24.7% O=C-O. The average hydrodynamic diameters (Hd) of GO (1–100 μg/L) in E3 embryo culture medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM MgSO4, pH 7.4) were approximately 180–360 nm.

2.2. Zebrafish maintenance and GO exposure

The zebrafish used in this study were wild-type AB-strain zebrafish (*Danio rerio*, 6 months of age) and were housed in a reverse osmosis system with a 14-h light/10-h dark cycle at 28 °C. Adult fish were fed freshly hatched brine shrimps twice per day. Embryos were acquired under natural breeding conditions; specifically, healthy embryos were collected immediately after fertilization and washed with E3 culture medium to remove any feces and infertile eggs. GO nanosheets were dispersed in E3 culture medium through sonication for 1 h at 100 W in ice water to prepare a stock suspension (1 mg/L). The stock suspension was diluted to 1, 10 and 100 μg/L with E3 culture medium under the same sonication conditions. The embryos in the treated groups were exposed to GO (1, 10 and 100 μg/L) in E3 culture medium from 2.5 h post-fertilization (hpf) to 7 days post-fertilization (dpf), and embryos treated with E3 culture medium without GO were used as the control group. Throughout the experiment, the embryos were incubated at 28 °C in a climate cabinet (SPX-300I-C, BOXUN, China) with a 14-h light/10-h dark cycle, and at 24-h intervals, the dead embryos were removed, and the exposure solution was renewed. Prior to renewal, all GO suspensions were dispersed ultrasonically at 28 °C for 30 min. All fish were handled humanely and were treated in accordance with the practices outlined by the Institutional Animal Care and Use Committee (IACUC), and the protocols were approved by the Animal Experiments Ethical Committee of Nankai University.

2.3. Toxicological experiments

Healthy embryos at 2.5 hpf were randomly transferred into 24-well plates (one embryo in a 2-mL suspension per well) and cultured for 7 days post-fertilization (dpf). The number of dead individuals and embryonic malformations were checked daily via light microscopy (Olympus ZL 61, Olympus, Tokyo, Japan). Mortality was identified by embryo coagulation, a stopped heartbeat and/or a lack of swimming. The specific endpoints (tail flexure, pericardial edema, yolk sac edema and head malformation) of the malformations were calculated at 7 dpf. For the mortality and malformation analysis, each treatment group included thirty larvae, and each treatment was performed in triplicate (90 larvae per concentration). The heart rate at 96 hpf was recorded by continuous observation for 1 min under a light microscope (Olympus ZL 61, Olympus, Tokyo, Japan). In this analysis, each treatment group included ten larvae, and each treatment was performed in triplicate (30 larvae per concentration). The alteration in the loss of mitochondrial membrane potential was measured by JC-1 fluorescence staining (Chen et al., 2015a, 2015b) at 7 dpf using a fluorescence microscope (Olympus X71; Olympus, Japan); six larvae were included in each treatment group, and each treatment was performed in triplicate (18 larvae per concentration). The glutathione (GSH) and Na^+/K^+-ATPase contents at 7 dpf were detected using a glutathione assay kit and a Na^+/K^+-ATPase assay kit provided by the Nanjing Jiancheng Bioengineering Institute, China, respectively, in accordance to the manufacturer's instructions. Twenty-four larvae were included in each treatment group, and each treatment was performed in triplicate (72 larvae per concentration).

2.4. Proteomic analysis

To reveal the specific mechanisms of GO toxicity, protein levels were analyzed through a LC-MS/MS-based proteomic method described previously (Sun et al., 2016). Because zebrafish exhibited developmental malformations after treatment with 1 and 100 μg/L GO, zebrafish exposed to 1 and 100 μg/L GO were selected for the protein expression analyses. Embryos were exposed to E3 culture medium, 1 μg/L GO or 100 μg/L GO. After exposure for 7 days, 600 larvae of each exposure group were homogenized on ice with lysis buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 100 mM KCl, 420 mM NaCl, 20% glycerol, 0.5 μg/L aprotinin, and 0.5 μg/L leupeptin; pH 7.4). After centrifugation at 15,000*g* and 4 °C for 15 min, the supernatant was recovered, and the total protein concentration was quantified using a Bradford protein assay with bovine serum albumin as the standard. Filter-aided sample preparation (FASP) was used for protein digestion with a 30-kDa molecular weight cutoff spin ultrafiltration filter.