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Comparative toxicity of pristine graphene oxide and its carboxyl, imidazole or polyethylene glycol functionalized products to *Daphnia magna*: A two generation study☆

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ABSTRACT

To investigate the chronic toxicity of graphene oxide (GO) and its functionalized products (GO-carboxyl, GO-imidazole and GO-polyethylene glycol), a two-generation study was conducted using the aquatic model species *Daphnia magna*. Each generation of daphnids were exposed for 21 days to 1.0 mg L^{-1} graphene material, with body length, neonate number, time of first brood and the intrinsic rate of natural increase (r) assessed as endpoints. Chronic exposure to GO, GO-carboxyl, and GO-imidazole had no adverse effect on body length or offspring number in the daphnid F0 generation, however, this exposure paradigm led to significant growth or reproduction inhibition in the following generation. Meanwhile, GO was found to show the strongest inhibitory effect, sequentially followed by GO-carboxyl and GO-imidazole. With exposure to GO-polyethylene glycol, no significant effects on growth or reproduction were observed for both F0 and F1 generation daphnids. These results reveal that carboxyl, imidazole and polyethylene glycol functional attachments alleviate the bio-toxicity of GO, especially polyethylene glycol. The increased C/O atomic ratio present in GO-carboxyl, GO-imidazole and GO-polyethylene glycol due to functionalization may mainly explain the reduced toxicity.

1. Introduction

Graphene is widely studied in nanotechnology because of its unique mechanical (Dreyer et al., 2009), electrical (Bolotin et al., 2008), optical (Dreyer et al., 2009), thermal (Balandin et al., 2008) and chemical properties (Dimitrakakis et al., 2008). Reported literature has demonstrated that graphene-based materials will induce a widespread and profound influence on a range of applications, including structural nanocomposites (Akhavan et al., 2010; Chang et al., 2012), battery electrodes (Kim et al., 2009b), supercapacitors (Stoller et al., 2008) and biomedicine (Bao et al., 2011; Liu et al., 2013). It is expected that the output and application scope of these materials will significantly increase over the next few years (Bianco, 2013). Nevertheless, the increased use of graphene-based materials will promote their discharge into the aquatic environment throughout the processes of manufacturing, transportation and consumption, leading to toxicity in aquatic ecosystems (Zhao et al., 2014). Therefore, toxicological studies are required to assess the health threat and environmental risk associated with exposure of aquatic organisms to graphene-based materials.

Various studies have focused on the toxicity of graphene-related materials to aquatic organisms. For example, pristine graphene has been reported to accumulate in the gut of *Daphnia magna* and be transferred to neonates after a 48 h exposure (Guo et al., 2013). Graphene grafted by TiO_2 nanoparticles was found to induce significant phototoxicity to *D. magna* and *Oryzias latipes* during a 48 h exposure (Li et al., 2014). Graphene oxide (GO) at concentrations of 100.0 mg L^{-1} resulted in significant oxidative stress and mitochondrial toxicity during zebrafish embryogenesis with a 69.5 h exposure (Chen et al., 2015b). In addition, alteration to the intestinal microbial community structure of adult zebrafish has been observed after exposure to few-layer graphene for 72 h (Lu et al.,

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2017). However, although the aquatic toxicity of graphene-related materials has received much research attention, most of the risk assessments have been based on short-term evaluations. Long-term pollutant exposure over the full life-span of multiple generations is the realistic exposure scenario due to the continuous discharge of pollutants into the environment (Minguez et al., 2015). Our earlier studies have found that graphene at concentrations of 0.5–1.0 mg L^{-1} can obviously inhibit the growth and reproduction of *D. magna,* following a 21 day exposure period, although no mortality was observed (Fan et al., 2016). Previous studies have also confirmed that graphene-based materials can induce changes to the epigenome of model organisms (such as DNA methylation, histone modifications, or downstream target gene up-regulation due to altered RNA expression) (Chen et al., 2016b; Jeong et al., 2015; Sasidharan et al., 2015), which would probably affect the next generations. Moreover, the transfer of effects of nanomaterial exposure to the next generation of organisms could potentially have a catastrophic effect on long-term population survival. A detailed mechanistic understanding of how graphene-based materials affect intergenerational population dynamics in model organisms, as well as the persistent effects following removal of pollutant materials, will advance our understanding of the long-term ecological impacts of exposure to graphene-based materials. Therefore, chronic toxicity studies using multi-generational exposure are essential to obtain a complete understanding of the long-term population effects of graphene-based materials.

Recent studies on GO have focused on tailoring its surface chemistry for enhancing its performance in bio-sensing and medical science (Li et al., 2017; Sahoo et al., 2011), which was highlighted by GO-carboxyl, GO-imidazole and GO-polyethylene glycol. It is of note that surface chemistry plays a significant part in determining the toxicity of graphene-based materials to biological receptors. Pristine graphene was reported to induce serious oxidative stress leading to apoptosis in monkey kidney cells, whereas carboxyl functionalized graphene induced no toxicity to these cells (Sasidharan et al., 2011). After injection, GO-polyethylene glycol persisted in the mouse body for an extended period of time, without exerting remarkable toxic effects on the treated animals (Yang et al., 2013). Based on these reported findings, it may be expected that altering surface functionalization consequentially changes the toxicity of graphene-based materials to biological receptors; however, the interaction between functionalized GO and aquatic organisms remain largely unknown, especially over a long-term period.

Herein, four graphene-based materials with differing surface functionalization (GO, GO-carboxyl, GO-imidazole and GO-polyethylene glycol) were dispersed in water and their chronic effects were investigated over two generations, using the aquatic organism *D. magna*, the standard biological model recommended by the U.S. Environmental Protection Agency. The survival, body length, time of first brood, neonate numbers and intrinsic rate of natural increase (r) were observed as adverse outcome end points to elucidate the toxic mechanism. The present study will contribute to our understanding of the long-term environmental risks of functionalized GO exposure, helping to guide their future applications.

2. Materials and methods

2.1. Organisms and medium

D. magna were maintained in unpolluted natural water obtained from the Jingmi River (116°16′ 732 E, 39°58′ 401 N) at a constant temperature of 23.5 °C with 16:8 (light-dark) photoperiod. Water was renewed every two days and the green algae *Chlamydomonas reinhardtii* were added as a daily food source. The simplified Elendt M7 medium (SM7), which consists of $CaCl_2$, $MgSO_4$, K_2HPO_4, $NaNO_3$, $NaHCO_3$, Na_2SiO_3, H_3BO_3, KCl, and KH_2PO_4, water hardness of 250 mg $CaCO_3$ L^{-1}, and pH of 7.8–8.2 recommended by standard testing guidelines (OECD, 2012) was select as the test medium and used in all experiments.

2.2. Chemical preparation and analyses

GO, GO-carboxyl, GO-imidazole and GO-polyethylene glycol were purchased from Nanjing XFNANO Materials Tech Co., Ltd. (Nanjing, China) with >99 purity and a 0.5–5 μm lateral size. These four graphene-based materials with different surface modifications were chosen as they represent the range of commercially available graphene-based materials that are currently showing promise for industrial application. To prepare stock solutions of GO, GO-carboxyl, GO-imidazole and GO-polyethylene glycol, 10 mg powdered materials were bath-sonicated in 100 mL SM7 medium for 2 h (100W, KQ-500DB, Kun Shan, China). During sonication, most of the materials were suspended and no precipitate was observed. Solutions were then left to settle for 24 h and the settled material was collected to verify the degree of dispersion according to the method by Kim et al. (2009a). The concentration of graphene-based materials in all supernatants were found to be higher than 90 mg L^{-1}, indicating that the materials were relatively well dispersed in SM7 medium. Therefore, a nominal concentration of 100 mg L^{-1} was applied as the estimated concentration of the final stock solutions. Stock solutions were stored at room temperature and were re-sonicated for 20 min immediately prior to dilution of working suspensions with SM7 medium for use in bioassays.

All graphene-based materials were characterized upon receipt from the supplier with composition analysis performed using X-ray photoelectron spectroscopy (XPS; Escalab 250, Al Ka, USA). The C1s XPS spectra were fitted and decomposed with XPSPEAK41 software. The peak area ratios of XPS core levels were analyzed, along with the sensitivity factor for each element, to determine the C/O atomic ratios of each material. Raman spectra were acquired on a HR-800 Jobin-Yvon instrument at an excitation wavelength of 532 nm. Surface topography, lateral size and height profiles were characterized for all graphene-based material sheets based on transmission electron microscopy (TEM, JEOL, 2010F) and atomic force microscopy (AFM, ICON, Veeco, USA) of aqueously dispersed samples.

In order to quantitatively assess the stability of GO, GO-carboxyl, GO-imidazole and GO-polyethylene glycol suspensions between water renewal periods during the toxicity test, the change of electrophoretic mobility (EPM), hydrodynamic diameter and concentration of the four materials in SM7 medium (1.0 mg L^{-1}) were monitored in the presence or absence of daphnids over a period of 48 h. Briefly, four 14-d-old daphnids were placed into 100 mL graphene-based material suspensions, with three replicates prepared for each material treatment. Meanwhile, three additional graphene-based material suspensions were prepared to serve as a control. Daphnids were fed with algae at 0 and 24 h (cell density = 10^5 cells/mL, similar to densities applied in chronic toxicity test), with the content of dissolved oxygen and light transmittance of the medium recorded over time. After exposure for 0, 2, 4, 8, 16, 32, 40 and 48 h, the electrophoretic mobility (EPM) of the four materials was measured using a Zetasizer Nano ZS (Malvern, UK). The average particle sizes of the four materials and their aggregates were established using hydrodynamic diameter, measured by dynamic light scattering (DLS). In addition, UV-VIS spectrophotometry (U3900H, Hitachi, Tokyo, Japan) was used to quantify the residual graphene-based materials in suspension at their peak wavelengths (235 nm for GO and GO-carboxyl; 205 nm for GO-