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Eco-friendly profile of pegylated nano-graphene oxide at different levels of an aquatic trophic chain

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ABSTRACT

Nanographene oxide (nGO) has been recently proposed as a new antitumoral therapeutic agent, drug delivery carrier and gene transfection vehicle, among others. Treatment is carried out by hyperthermia induced by infrared irradiation. After treatment, the nanosystems will be inevitably excreted and released to the environment. To understand the potential impacts of pegylated nGO (nGO-PEG), three key species from different trophic levels were used: the green micro-algae *Raphidocelis subcapitata* (growth inhibition test), the cladocera *Daphnia magna* (acute and chronic tests), and the fish *Danio rerio* (fish embryo test). Besides a regular standard procedure to assess toxicity, and considering the mode of action of nGO-PEG in cancer treatment, a simultaneous infrared lamp exposure was carried out for *D. magna* and *D. rerio*. Additionally, and taking advantage of the phenotypic transparency of *D. magna*, nGO-PEG was fluorescently tagged to evaluate the potential uptake of nGO-PEG. The *R. subcapitata* growth inhibition test showed effects during the first 48 h, recovering till the end of the test (96 h). No acute or chronic effects were observed for *D. magna*, under standard or infrared light exposures although confocal microscope images showed nGO-PEG uptake. Very small percentages of mortality and abnormalities were observed in *D. rerio* exposed with and without the infrared lamp. Although low hazard may be expected for nGO-PEG in aquatic ecosystems, further studies with species with different life traits should be accomplished, in order to derive more accurate conclusions.

1. Introduction

Nanotechnology is providing tools to solve global, crucial and longstanding problems regarding environmental, agricultural, energetic and medical applications. Medical applications using nanotechnological methodologies are sound and auspicious for enduring problems like cancer, providing a more efficient drug delivery and treatments with less secondary effects to patients.

As described above, nanographene oxide (nGO) has been developed for several purposes: nanosheets for corrosion-inhibiting coating (Krishnamoorthy et al., 2013), nano-fillers for cement in constructions (Chuah et al., 2014), transparent conductive films for electronics, among others. Besides the inherent properties of nGO as a nanomaterial, providing a small size and large surface area, its functionalization is an add-value for medical requests acting as a smart material with low cytotoxicity to healthy cells. Nano-GO can be hybridized with polymers, magnetic and gold nanoparticles, widening the range of bio-applications, from photothermal and photodynamic therapies to imaging techniques and drug delivery or antibacterial roles (Nanda et al., 2015).

In 2012, Vila et al. described PEG functionalized GO nanosheets which effectively entered osteoblast-like cells without inducing high membrane damages, crucial when testing biomaterials. These pegylated GO nanosheets have also been described as a weak inflammatory biomaterial, another important characteristic concerning hyperthermia cancer therapy (Feito et al., 2014; Goncalves et al., 2014; Vila et al., 2014). The therapy is based on the energy transfer due to nGO's optical absorption capacity in the near infrared region (NIR 700–1100 nm) causing cell death by hyperthermia and tumor destruction above 43 °C. Along with their high treatment efficacy, the pegylated GO nanosheets do not change their characteristics after metabolization and/or excretion by the human system (Yang et al., 2010). Therefore, and following the life-cycle of materials, requested by several international legislations, it is essential to understand the potential impacts of this

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nanomaterial to the environment. Although not much information is available on the changes that nGO may undergo in a waste water treatment plant, it has been described that at high concentrations (50 and 300 mg/L) nGO induces acute toxicity (Ahmed and Rodrigues, 2013), mainly related to bacterial metabolic activity, bacterial viability, and biological removal of nutrients (e.g. organics, nitrogen and phosphorus) (Deng et al., 2017). Although no predicted environmental concentrations (PEC) for nGO can be found in the literature, one cannot expect these effect levels (50 and 300 mg/L) to be environmentally relevant. In the approach carried out by Mesaric et al. (2015) to predict risk to carbon-based nanomaterials, PEC values for carbon black were established based on values for heavily contaminated freshwater within 0.08–7.5 mg/L. Regarding carbon nanotubes, the PEC was established to concentrations up to 67 ng/L. Therefore, studies with environmentally relevant concentrations should be carried out, based on the assumptions that higher concentrations of carbon-based nanomaterials tend to present higher aggregation/agglomeration rates, potentially leading to an underestimation of effects that could be perceived under lower concentrations (Lanphere et al., 2014). In addition, understanding the mode of action of a nanomaterial synthetized for a specific purpose is also essential to establish a relevant experimental design and approach.

Evidences for graphene based compounds accumulation in several organisms have been reported to be dependent on the material's properties. In the study of Lu et al. (2017), while large carbon-14-labelled few-layer graphene (FLG; 250 μg/L) accumulated in the zebrafish gut, smaller FLG accumulated in the liver, showing evidences that FLG can pass the gut barrier. Similarly, *Daphnia magna* exposed to FLG was also dependent of the material type, and a decrease in the FLG bioaccumulation and toxicity was observed after being modified by using a natural organic matter transformation processes mediated by horseradish peroxidase (HRP) mediated reactions (Lu et al., 2015). Besides previous findings, it was also observed that the presence of cladocera increased the aggregation and settling of FLG after a 48 h exposure to 250 μg/L (approx. 80%) possibly related to FLG's gut passage, increasing concentration in the digestive system and posterior release to the media (Guo et al., 2013).

Therefore, the present study aimed to assess the hazard of a functionalized nGO, synthetized for medical purposes, to freshwater ecosystems. For that, nGO's toxicity was assessed in three key species, from three different trophic levels: the green micro-algae *Raphidocelis subcapitata*, the cladocera *Daphnia magna*, and the fish *Danio rerio*. Additionally, besides the traditional laboratory acute bioassays, co-exposures of nGO-PEG under infrared light (1000–1200 nm) were carried out with *Daphnia magna* and *Danio rerio* to understand this specific material related effects, i.e. considering the absorbance of GO in the near-infrared wavelength region (key feature for performing the hyperthermia treatments). Furthermore and by taking advantage of the phenotypic transparency of *D. magna*, nGO was fluorescently tagged to visually confirm the potential entrance and real exposure of the test-organism to nGO.

2. Experimental methodology

2.1. Functionalized PEG nanographene oxide

The functionalization of nanographene oxide by covalent bonding with the non-toxic and non-immunogenic polymer poly (ethylene glycol-amine) (PEG) was carried out following the procedure reported by Vila et al. (2012). Briefly, GO nanosheets were obtained from graphite by a modified Hummers method and further ultrasonic treatment in distilled water. In order to promote the carboxylation of the GO sheets, chloroacetic acid and sodium hydroxide (5% m/v each) were added to an aqueous dispersion of GO (~ 1 mg/mL) and sonicated for 3 h. Once neutralized and washed, the GO-COOH sheets were covalently functionalized with PEG (poly-ethylene glycol bis (3-aminopropyl) terminated, Mn ~ 1500 Da, Sigma-Aldrich) using EDC as zero-length crosslinker (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Sigma-Aldrich). The aqueous mixture was stirred for 48 h at room temperature and subsequently washed by centrifuge filtration (Amicon Ultra 100 kDa MWCO). This methodology confers decreased aggregation as well as avoidance of intercession with cellular functions or target immunogenicity (Cicuendez et al., 2017).

To obtain fluorescein labelled nGO, the pegylated nGO was stirred overnight with the amine reactive dye FITC (Fluorescein isothiocyanate, Sigma) in PBS buffer, and then washed by centrifugation to remove the non-attached dye molecules (Feito et al., 2014).

2.2. Nanographene oxide characterization

Electron microscopy of the nGO particles (without PEG) was performed on a 200 kV Transmission Electron Microscope, HR-(EF)TEM - JEOL 2200FS, equipped with a B-U Oxford INCA Energy TEM 250 EDS spectrometer for elemental analysis. A few drops of ultrasonicated nGO solution in MQ water, were dropped on top of the carbon copper grid and was dried before TEM observation. nGO-PEG was characterized in all media used in the ecotoxicity tests using a suspension of 1 mg/L, at time 0 h and at the end of the test or media renewal (48 h for ASTM and 96 h for ultrapure water, MBL (Marine Biological Laboratory) and fish system water). The size of the nGO's particles was measured by dynamic light scattering analyses (DLS) using a Zeta Sizer Nano Series (Malvern), at 25 °C. DLS data were assumed as the average hydrodynamic size with a diameter corresponding to the lateral length of the nanosheets. The zeta potential, providing information on the surface charge over time, was also measured.

2.3. Test organisms

The green microalgae *Raphidocelis subcapitata* was maintained in Woods Hole MBL culture medium, in controlled laboratory conditions: continuous light (visible) and temperature of 20 °C ± 1 °C. Exponential growing cells were used for the bioassays and 7-days old cultures were renewed by inoculation in a new MBL medium.

Daphnia magna (Beak clone) cultures were maintained in ASTM (American Society for Testing and Materials) medium in controlled laboratory conditions: temperature 20 ± 1 °C; photoperiod 16/8 h light/dark cycle (visible light). Media was renewed and the daphnids fed every other day with the micro green algae *R. subcapitata*, at a concentration of 3×10^5 algae cells/mL. A seaweed extract was also added to the culture medium as a supply of organic matter and micronutrients. Neonates between the third and fifth brood (< 24 h) were collected and used in each toxicity test.

Zebrafish (*Danio rerio*) eggs were provided from the culture established at the Biology Department from the University of Aveiro (Portugal) maintained in laboratory controlled conditions: carbon-filtered water at 26 ± 1 °C, photoperiod cycle of 16/8 h light/dark (visible light); conductivity of 750 ± 50 μs; pH 7.5 ± 0.5; dissolved oxygen at 95% saturation; fed with commercial artificial diet (ZM 400 Granular) and brine shrimp twice a day.

2.4. Toxicity tests

The initial nGO-PEG dispersion was kept in continuous shaking after being synthetized and until used in the ecotoxicological assays. For each bioassay, test dispersions were prepared immediately before use by dispersion in the respective medium of each organism used for testing.

2.4.1. Raphidocelis subcapitata

The algae growth inhibition test with *R. subcapitata* was adapted from the OECD (The Organisation for Economic Co-operation and Development) 201 guideline (OECD 2006) and the methodology