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Deciphering the underlying mechanisms of oxidation-state dependent cytotoxicity of graphene oxide on mammalian cells

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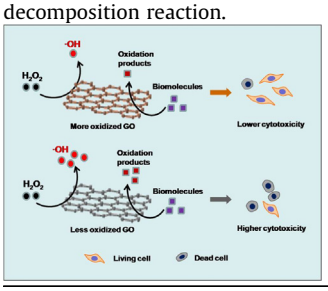
HIGHLIGHTS

- A strong association between the oxidation level and toxicity of GO was found.
- A systemic chemical mechanism of GO cytotoxicity is proposed.
- ROS conversion facilitation and the oxidative ability of GO are attributable.
- The key role of carboxyl groups in varying the energy barrier of ROS reaction.
- Both experiments and theoretical simulation were conducted.

GRAPHICAL ABSTRACT

The cytotoxicity of three GOs with different oxidation degrees, GO-h, m and l (GO-high, medium and low) were tested on mouse embryo fibroblasts (MEFs). Significantly higher level of oxidative stress in cells was induced by the GO with lower oxidation degree in relation to its stronger toxicity to cells. This can be attributed to its stronger indirect oxidative damages through facilitating ROS conversion and higher direct oxidative abilities on intracellular biomolecules. Theoretical calculation indicated the key contributions of oxygenation-level-based nanostructure to varying the energy barrier of H_2O_2 decomposition reaction.



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ABSTRACT

The promising broad applications of graphene oxide (GO) derivatives in biomedicine have raised concerns about their safety on biological organisms. However, correlations between the physicochemical properties, especially oxidation degree of GOs and their toxicity, and the underlying mechanisms are not well understood. Herein, we evaluated the cytotoxicity of three GO samples with various oxidation degrees on mouse embryo fibroblasts (MEFs). Three samples can be internalized by MEFs observed via transmission electron microscopy (TEM), and were well tolerant by MEFs at lower doses (below 25 μg/ml) but significantly toxic at 50 and 100 μg/ml via Cytell Imaging System. More importantly, as the oxidation degree decreased, GO derivatives led to a higher degree of cytotoxicity and apoptosis. Meanwhile, three GOs stimulated dramatic enhancement in reactive oxygen species (ROS) production in MEFs, where the less oxidized GO produced a higher level of ROS, suggesting the major role of oxidative stress in the oxidation-degree dependent toxicity of GOs. Results from electron spin resonance (ESR) spectrometry showed a strong association of the lower oxidation degree of GOs with their stronger indirect oxidative damage through facilitating H_2O_2 decomposition into •OH and higher direct oxidative

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abilities on cells. The theoretical simulation revealed the key contributions of carboxyl groups and aromatic domain size of nanosheets to varying the energy barrier of H_2O_2 decomposition reaction. These systematic explorations in the chemical mechanisms unravel the key physicochemical properties that would lead to the diverse toxic profiles of the GO nanosheets with different oxygenation levels, and offer us new clues in the molecular design of carbon nanomaterials for their safe applications in biomedicine.

1. Introduction

The potential nanotoxicity of graphene and its derivatives (such as graphene oxide, GO) has received increasing attention due to their great range of potential applications, especially in biomedical fields, such as biosensors (Akhavan et al., 2012a), photothermal therapy (Sun et al., 2008) and drug delivery (Zhang et al., 2011). Although various covalent and non-covalent methods for GO functionalization have improved their stability and biocompatibility, and even reduced their toxicity in physiological environments (An et al., 2013; Liu et al., 2010; Pan et al., 2012), GO itself still occupies the most important position in the initial molecular design process. We have to face the inevitable issue to what oxidation degree we should "oxidize" graphite, the initial source to synthesize GO. Therefore, to evaluate the safety of GO on biological organisms, unraveling the association between the toxicity and the oxidation degree of nanosheets becomes an urgent task.

So far there have been a number of studies regarding the biological effects of GO and reduced GO (rGO) (or pristine graphene) on models like mammalian cells (Das et al., 2013; Sasidharan et al., 2011) and bacteria (such as *E. coli*) (Liu et al., 2011; Akhavan and Ghaderi, 2010) as well as mice (Wang et al., 2011) and *C. elegans* (Zhang et al., 2012). However, the researchers have not reached to consensus on the correlation between oxidation degree of nanosheets and their toxicity. More importantly, the previous reports regarding chemical mechanisms underlying the toxicity of GO samples with different oxygenated functionalization are still limited (Das et al., 2013; Liu et al., 2011, 2012). One point held that GO is more toxic than rGO. As for mammalian cell models, GO was found to be more toxic than rGO on human umbilical vein endothelial cells (HUVEC) (Das et al., 2013). It was hypothesized that more reactive functional groups (e.g. —OH, —COOH) of GO would have a greater potential to interact with biological macromolecules compared with reduced GO, emphasizing the favor of oxygenated groups on enhancing the bio-nano interaction efficiency. As for bacterial models, both membrane damage and oxidative stress were proposed to be critical to GO toxicity (Liu et al., 2011), and the smaller size and better dispersion of more oxidized GO in physiological conditions might be responsible for their higher antibacterial activity on *E. coli*. Contrarily, it has also been reported that rGO processed higher toxicity than GO on mammalian cells (Sasidharan et al., 2011) and bacteria (Akhavan and Ghaderi, 2010). Sasidharan et al. (2011) reported that large accumulation of pristine graphene on the monkey kidney cell membrane induces intracellular reactive oxygen species (ROS) production thus leading to apoptosis, while carboxyl functionalized graphene allowed cells to function relatively normally, though internalized by the cells. Akhavan and Ghaderi (2010) proposed that the higher antibacterial effects of the reduced GO might be attributed to the better charge transfer between the bacteria and the more sharpened edges of the reduced GO during the contact interaction. Besides, some researchers also found both GO and rGO solid substrates were biocompatible to NIH-3T3 cells (Ryoo et al., 2010). The discrepancy may be attributed to different sample properties and biological models.

In this work, we synthesized three GO samples, GO-h, GO-m and GO-l (GO-high, GO-medium and GO-low), all with good solubility in water and similar sizes but distinct oxidation levels. By this we aimed to minimize the interference of size and solubility when evaluating the correlation between the oxidation state and the toxicity of GO. We investigated their effects on the internalization, morphology, viability and apoptosis of mouse embryo fibroblasts (MEFs) in relation to both the intracellular and in vitro ROS generation by GOs. Based on results from electron spin resonance (ESR) spectrometry and theoretical simulation, we present a mechanism showing a strong correlation between the oxidation-degree-based nanostructures of GO and their cytotoxicity, and for the first time interpret the chemical mechanisms involved in the different toxicological behaviors of three GO samples with distinct properties on oxidation degree.

2. Materials and methods

2.1. Preparation of GO with various degrees of oxidation

Natural graphite was purchased from Alfa Aesar. $KMnO_4$, concentrated H_2SO_4 and H_2O_2 (30%) were obtained from Beijing Chemical Reagent Co. All the chemicals were of analytical grade and used without further purification. Deionized water was used throughout the experiment. Graphite oxides with various degrees of oxidation were prepared from graphite flake using the reported modified Hummer's method (Hummers and Offeman, 1958; Yan et al., 2013). In brief, 0.2 g of graphite was stirred in 10 ml of concentrated H_2SO_4 for 5 h. The required amount of $KMnO_4$ was then gradually added to the above solution in ice-bath while keeping the temperature at less than 4 °C. The obtained mixture was stirred at 37 °C for 2 h. Afterwards, 20 ml of deionized water was added under vigorous stirring to dilute the resulting solution. The suspension was further treated by adding H_2O_2 solution (10 ml) to remove the residual $KMnO_4$ and MgO_2. The acid washed paste was filtered and washed with 10% HCl solution and deionized water until the pH value of the solution became neutral. Finally, the resulting graphite oxide was subjected to dialysis for 7 days to remove residual metal ions and acid. The cut off size of the dialysis membrane was 3500 D and the medium used was deionized water (~18.2 MΩ/cm). The dialysis medium was refreshed every 3 h for the first day and daily for the last 6 days. The GO nanosheets were obtained by diluting the resulting graphite oxide suspension with deionized water until the concentration reached to 1 mg/ml, and then the above suspension was probe-sonicated at the power of 325 W for 4 h, followed by centrifuging at 1,760 *g* for 20 min to remove any unexfoliated graphite oxide. The degree of oxidation was tuned by changing the amount of $KMnO_4$ from 0.3 g to 0.7 g with an increment of 0.2 g per oxidation level while other parameters were kept constant in the reaction. Before use, three GO liquid samples (dispersed in water) were sonicated using an ultrasonic cleaner (KQ2200E) at 100 W for 15 min.

2.2. Characterization of GO samples

Atomic force microscopy (AFM) images were taken on a scanning probe microscopy (SPM, Multimode 8, Bruke) under ambient conditions. The micro-Raman spectroscopy (Renishawin Via Raman Spectroscope) experiments were performed under