Original Article

Specific nanotoxicity of graphene oxide during zebrafish embryogenesis

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Abstract

Graphene oxide (GO) has shown great potential for biological, medical, energy and electronic applications. As a consequence of these diverse applications, GO release into the ecosystem is inevitable; however, the corresponding risks are largely unknown, particularly with respect to the critical period of embryogenesis. This study revealed that GO adhered to and enveloped the chorion of zebrafish embryos mainly via hydroxyl group interactions, blocked the pore canals of the chorionic membrane, and caused marked hypoxia and hatching delay. Furthermore, GO spontaneously penetrated the chorion, entered the embryo via endocytosis, damaged the mitochondria and primarily translocated to the eye, heart and yolk sac regions, which are involved in the circulatory system of zebrafish. In these organs, GO induced excessive generation of reactive oxygen species and increased oxidative stress, DNA damage and apoptosis. Graphene oxide also induced developmental malformation of the eye, cardiac/yolk sac edema, tail flexure and heart rate reduction. In contrast to the common dose-effect relationships of nanoparticles, the adverse effects of GO on heart rate and tail/spinal cord flexure increased and then decreased as the GO concentration increased. These findings emphasize the specific adverse effects of GO on embryogenesis and highlight the potential ecological and health risks of GO.

Introduction

Due to their unique physiochemical properties, two-dimensional graphene oxide (GO)-based materials have attracted considerable attention in various research communities and industries (Hu & Zhou, 2013; Li et al., 2013a; Wu et al., 2011). Reports project that the market for GO-based products will reach $675 million by 2020 (Ahmed & Rodrigues, 2013). Unfortunately, while the increased use of GO-based materials promotes their release into the environment, the health risks associated with environmental exposure to GO are largely unknown (Hu & Zhou, 2013), particularly with respect to embryogenesis (George et al., 2014). A few studies of GO nanotoxicity have been conducted in mammals (Li et al., 2013a), biomacromolecules (Mahmoudi et al., 2012; Wu et al., 2011), bacteria and different cell lines (Hu et al., 2011; Zhang et al., 2013). Use of in vitro cell culture to initially assess the toxicity of nanomaterials is fairly simple and cost-effective; however, it is nearly impossible to imitate an in vivo system, and several controversial results have been reported (Ashkarran et al., 2012; Peralta-Videa et al., 2011). Although small mammalian models remain the standard method for assessing the possible toxicity and distribution of nanomaterials in humans, establishing mammalian models is often expensive and time-consuming (Fako & Furgeson, 2009; Zhou et al., 2004). Thus, zebrafish (Danio rerio) have been established as a preferred vertebrate model for studying the toxicity of nanomaterials in vivo due to the close homology between zebrafish and human genomes (Wiecinski et al., 2013). In addition, zebrafish and humans exhibit similar physiological responses, especially during the development of chronic disease (George et al., 2014; Wiecinski et al., 2013; Zhou et al., 2004).

Embryogenesis is an important phase of life, and dysfunctions in embryogenesis are associated with various diseases and adverse effects, such as hypoxia, malformation, organ function and eccyliosis (Chen et al., 2013; Ilan et al., 2013; McGee et al., 2012). The zebrafish embryo is a unique model system, which is used to investigate the effects of nanoparticles on embryogenesis (Heiden et al., 2007); however, three critical issues have thus far been ignored. First, compared with other nanomaterials, GO displays excellent mechanical and hydrophilic properties while remaining highly flexible and ductile (Dikin et al., 2007). These properties enable GO to adhere to a single cellular surface (Sanchez et al., 2012; Shi et al., 2012). Furthermore, recent research has shown that GO can envelop living microorganisms (Akhavan et al., 2011; Wahid et al., 2013); however, the envelopment of large embryos by GO and the adverse effects

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of this process remain unclear. Second, in contrast to common nanoparticles, and despite a thickness of only approximately 1 nm, GO nanosheets are quite large, ranging from dozens of nm to several μm in length (Dikin et al., 2007; Hu et al., 2011). As a result, the real-time dynamics of GO permeation of embryos and translocation to specific organs may be unique. Third, compared with carbon nanotubes (CNTs) and fullerene, the structure of two-dimensional GO nanosheets determines their fate and biocompatibility in vivo (Li et al., 2013a; Su et al., 2010). The specific envelopment and translocation properties of GO may induce unique nanotoxicity and should be thoroughly explored.

The primary goals of the present study were to characterize the interactions between GO nanosheets and zebrafish embryos and to analyze the localization and adverse effects of GO in vivo. Specifically, we determined the following properties: (i) GO envelopment of the chorion of developing embryos and its hypoxic effects in this context; (ii) the translocation of GO to specific organs of embryos; and (iii) the relationships among excessive generation of reactive oxygen species (ROS), apoptosis, DNA damage and developmental malformation.

**Methods**

**GO characterization**

The current environmental concentrations of GO are unknown. To enable a direct comparison with the doses of carbon nanomaterials reported in toxicity tests (Schwab et al., 2011; Serag et al., 2011), GO suspensions were prepared at different concentrations (100, 10, 1.0, 0.1 or 0.01 mg/L) in E3 embryo culture medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄; pH 7.4). GO nanosheets (purity >99%, single layer ratio ~99%) were obtained from Nanjing XFNANO Materials Tech Co., Ltd. (Nanjing, China). A 100 mg/L aqueous stock solution of GO was obtained by dissolving 100 mg of GO in 1000 mL of E3 solution followed by sonicication for 1 h. This GO solution was then diluted with E3 solution to produce graded concentrations of GO from 0.01 to 100 mg/L. Atomic force microscopy (AFM) images were acquired in tapping mode using an Agilent 5420 AFM instrument (Agilent, Santa Clara, CA). The size distribution and Zeta potential were characterized at a constant pH value (pH=7.4), a 30-mW 635-nm laser (Brookhaven, NY). The UV-vis spectra of all samples were recorded on a T90 spectrophotometer (Purkinje General, Beijing, China) equipped with UVW5 software in a 1-cm path-length quartz cuvette at 200–800 nm. Fourier transform infrared spectra (FT-IR) were recorded on a Bruker Tensor 27 infrared spectrometer with a resolution of 2 cm⁻¹ at 4000–4000 cm⁻¹. Energy-dispersive spectroscopy (JEOL, JEM-2010 FEF, Phoenix 60T, Tokyo, Japan) was performed to detect the elements that compose GO.

Zebrafish maintenance and the exposure of embryos to GO are described in the Supplementary material.

**GO-enveloped embryos and the hypoxic microenvironment**

To investigate the envelopment of embryos by GO, scanning electron microscopy (SEM) was conducted using a Hitachi SU8010 microscope (Hitachi, Tokyo, Japan). Further details are presented in the Supplementary material. To investigate the effects of GO on the functional groups of chorions, chorions were separated from embryos and analyzed by FT-IR. To measure the hypoxic microenvironment of the embryos, the oxygen concentrations were measured using a Unisense oxygen microsensor. Detailed methods are presented in the Supplementary material.

**Tracing the translocation of GO**

Graphene oxide (10 mL at 100 μg/mL) and fluorescein isothiocyanate (FITC) (100 μL at 1 mg/mL) were mixed under sonication for 10 min. The FITC-labeled GO (GO-F) solution was filtered using a dialysis membrane (Solarbio, MWCO 3.5–5 KD, Beijing, China) to remove the free FITC. Finally, time monitoring of GO-F transport in 24-hours-post-fertilization (hpf) embryos was performed using laser scanning confocal microscopy (LSCM) (Leica, TCS SP5, Wetzlar, Germany). Details regarding the analysis method are presented in the Supplementary material. To confirm the translocation and distribution of GO in embryos, paraffin and ultrathin sections were generated, and microstructure and ultrastructure observations were performed using optical microscopy (Olympus X71; Olympus, Tokyo, Japan) and transmission electron microscopy (TEM) (Hitachi HT7700, Tokyo, Japan), respectively. Details regarding the analysis method are presented in the Supplementary material. To identify GO deposits in the embryos, Raman spectra were recorded using a 514 nm laser (DXR Microscope, Thermo Scientific, Waltham, MA).

**Mortality, hatching, heart rate, spontaneous movement and malformation**

Embryos were analyzed daily, and the survival rate and alterations in morphology and toxicity endpoints were recorded (Choi et al., 2010). The E3 solution (200 μL) was replaced every 24 h until 96 hpf. The mortality of embryos or juvenile fish was determined by examining their movement, heartbeat and blood circulation via light microscopy (Olympus ZL 61, Olympus, Tokyo, Japan), and the results were recorded daily. At 48 hpf, the heartbeat and spontaneous movement of the embryo were recorded. At 72 and 96 hpf, hatching and the presence of physical abnormalities were recorded, respectively. Each experiment was performed in triplicate. The details of the analysis method are presented in the Supplementary material.

**Apoptosis, ROS and oxidative stress measurements**

The apoptotic rate was quantified in zebrafish embryos that had developed beyond 96 hpf according to the method previously described by Cheng et al. (2007). The generation of ROS in vivo was measured using the dichlorofluorescein diacetate method. Fluorescence was observed using a fluorescence microscope (Olympus X71; Olympus, Tokyo, Japan). The details of the analysis method are presented in the Supplementary material. Based on the above experiment, embryos exposed to 1 mg/L GO were observed, and apoptosis and ROS generation in specific regions were noted. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were measured using a TU-1901 spectrophotometer as previously described (Chio et al., 2012).

**Genotoxicity**

Genomic DNA was isolated from embryos developed over 96 hpf using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Concentrations of 8-hydroxy-2-deoxyguanosine (8-OHdG) in the zebrafish were determined by enzyme-linked immunosorbent assay as described in the Supplementary material. DNA methylation was measured via high-performance liquid chromatography (HPLC) (Waters 2695, Charlotte, NC) using a 250 x 4.6-mm Hyperclone C18 column. The percentage of global DNA methylation was quantified according to the Ramsahoye method (Ramsahoye, 2002).
**Statistical analyses**

To quantify GO in the embryos, aggregations of GO observed in TEM images were quantified using Image J software (NIH, Bethesda, MD). The relative fluorescence intensity was quantified using Olympus FV-10 ASW 3.1 software. IBM SPSS 22.0 statistical software (IBM SPSS, Chicago, IL) was used for all statistical analyses. Each experiment was performed at least in triplicate unless otherwise noted. The data were evaluated for homoscedasticity using Levene’s median test, and the one-way analysis of variance was employed to identify the effects that were associated with the exposure conditions and treatments. Dunnett’s test was used for pairwise multiple comparisons. The level of significance for all analyses was $p < 0.05$.

**Results**

**GO characteristics**

The characteristics of GO in E3 solution were examined in detail in the present study. We detected a peak at 230 nm and a peak shoulder from approximately 280 to 300 nm, which corresponds to the typical UV absorption spectrum of GO (Figure S1). No clear alteration of the UV spectra was detected after GO was maintained in E3 solution for 7 d. However, in ultrapure water, the zeta potential was $-30.5 \text{ mV}$, and this value increased to $-20.1 \text{ mV}$ in E3 solution (Figure 1A). TEM and SEM images revealed irregular folds in the GO nanosheets, which reflected the flexibility of GO (Figure 1D and E). AFM imaging demonstrated that the thickness of the GO nanosheets was approximately 0.8–1 nm (Figure 1B and C), and the diameter ranged from approximately 101 to 258 nm (centered on 147 nm), which was measured via wide-angle light scattering (Figure 1F). The FT-IR spectrum of GO clearly shows the presence of $\text{C}=\text{O}$ ($1720 \text{ cm}^{-1}$), $\text{C} = \text{C}$ ($1620 \text{ cm}^{-1}$), $\text{C}$–$\text{OH}$ ($1400 \text{ cm}^{-1}$) and $\text{C}$–$\text{O}$–$\text{C}$ ($1070 \text{ cm}^{-1}$) functional groups (Huang et al., 2014) (Figure 1G). The above results verify that the nanomaterial used is typical GO. In addition, 86.44% C, 12.74% O, 0.02% S, 0.01% K and 0.79% Cu (mainly contributed by the copper grid) were recorded by atomic percent-age in the EDX images (Figure S2).

GO initially enveloped chorions, inducing a hypoxic microenvironment

The optical microscopy images in Figure 2(A) show that the zebrafish embryos were transparent in the control group without GO exposure. However, yellow GO deposits appeared outside the chorion in the embryos exposed to GO, suggesting that the GO adsorbed to the chorion. This phenomenon was confirmed based on optical images of juvenile fish that displayed adherence to many deep yellow flakes (GO) after the embryos were exposed to GO (Figure S3). Characteristically, SEM revealed that the outer layer of the embryonic chorion was covered with protuberances of random sizes (Figure S4). Furthermore, SEM confirmed that the GO covered the chorion (Figure 2B). The envelopment by GO caused alterations to the functional groups of the chorion, such as an increase in C–OH ($1400 \text{ cm}^{-1}$) and a decrease in C–O ($1700 \text{ cm}^{-1}$) in the chorion after the embryos were exposed to 1 mg/L GO (Figure S5).

The concentration of oxygen near the chorion was 271 $\mu\text{M}$, and it gradually decreased to 264 $\mu\text{M}$ in the chorionic space when the sensor was inserted into the embryo to a depth of 250 $\mu\text{m}$ in the control sample (Figure 2C). For the embryos exposed to 1, 10 and 100 mg/L GO, the concentrations of oxygen shifted to 244, 243 and 227 $\mu\text{M}$, respectively, at a depth of 250 $\mu\text{m}$, which represented significant decreases ($p < 0.05$) compared with the control group (Figure 2C). In addition, there were no significant differences in the oxygen concentrations between the dechorionated embryos treated with GO and the control (Figure S6). These data

Figure 1. Characterization of GO. (A) Zeta potential of GO dissolved in ultrapure water and E3 solution. Data points correspond to mean values (two replicates; 6 measurements per replicate). (B and C) AFM images showing the thickness and diameter of GO; the arrows denote defects on the surface of GO (C). (D) TEM image. (E) SEM image. (F) The size of GO measured using a ZETAPALS/Bi-200SM instrument. Data points correspond to mean values (two replicates; 6 measurements per replicate). (G) FT-IR spectra of GO. AFM: atomic force microscopy; TEM: transmission electron microscopy; SEM, scanning electron microscopy; FT-IR, Fourier transform infrared spectra.
demonstrated that envelopment by GO caused hypoxia in the embryos.

**Uptake of GO and mitochondrial damage in zebrafish embryos**

To examine the transport of GO into embryos, zebrafish embryos older than 24 hpf were incubated with GO-F and then directly observed via LSCM. No fluorescence was observed in the embryos at the beginning of the GO-F incubation period (Figure 3A). After 8 min, fluorescence appeared around the embryo, particularly near the yolk sac (Figure 3A), suggesting that GO entered the embryos and selectively localized in the yolk sac. TEM also revealed that GO adsorbs to the surface of the chorion, permeates the chorion (Figure 3B), and localizes around...
the yolk sac (Figure S7). Many vesicles containing GO aggregates were observed in the embryos treated with GO, and these vesicles were not present in the control group (Figure 3B2–4). In addition, the uptake of GO did not increase with the GO concentration, and uptake was reduced in the embryos treated with 10 or 100 mg/L GO compared with the embryos treated with 1 mg/L GO (Figure S9).

Paraffin sections were prepared to examine the localization of GO in zebrafish embryos at 96 hpf, and it was found that GO primarily aggregated around the eyes, heart, yolk sac and tail (Figure S10). Moreover, embryos were transected into three sections at 96 hpf (Figure S11), and TEM was performed to confirm the localization of GO in these regions of the embryos (Figure 3C). It was found that a larger amount of GO aggregated in the chest compared with the head or tail (Figure 3D). In addition, mitochondria were regularly dispersed throughout embryo cells and exhibited a compact structure with complete membranes in the control groups (Figure 3E). However, the
membranes became swollen and loose and their integrity was compromised when the embryos were exposed to 1 mg/L GO for 8 h (Figure 3F), which indicated that the uptake and localization of GO may cause mitochondrial damage in zebrafish embryos. The typical D and G bands in the Raman spectrum (RS), which reflect the disordered structure and ordered $sp^2$ carbon structure of GO, were detected at approximately 1355 and 1602 cm$^{-1}$, respectively (Gollavelli & Ling, 2012). In the present study, the D and G bands were detected in the embryos treated with GO (Figure 3G), which confirmed that the above aggregates contained GO.

**Effects of GO on mortality, hatching, heartbeat, movement and morphology**

Mortality did not significantly increase in any group of GO-treated embryos with respect to the non-GO-treated control group (Figure S12). Similar results were obtained based on the hatching rates of embryos exposed to 0.01–10 mg/L GO (Figure 4A). However, compared with the control, a severe hatching delay was detected in the embryos treated with 100 mg/L GO: less than 30% of these embryos hatched after 96 hpf (Figure 4A). A reduced heart rate was observed in most of the GO-exposure groups, except for embryos exposed to GO at 100 mg/L at 48 hpf. The heart rate was significantly decreased in embryos treated with 1 mg/L GO compared with the control group; however, it was increased in 100 mg/L GO-treated embryos (Figure 4B). Moreover, spontaneous movement of embryos treated with 100 mg/L was significantly inhibited compared with that of the control embryos at 48 hpf (Figure 4C). Interestingly, the oxygen concentration in the chorion near the embryos was significantly positively correlated with the spontaneous movement of embryos at 48 hpf ($R^2 = 0.84$) (Figure S13).

GO caused several developmental malformations in the embryo at 96 hpf, including pericardial/yolk sac edema, tail/spinal cord flexure and no eyes/head, as determined at 96 hpf. Each experiment was conducted three times with $n = 40$ replicates. *$p < 0.05$ compared with the control.

GO caused apoptosis in the malformed organs

Apoptosis was markedly increased in the eye, heart and tail regions of GO-exposed embryos at 96 hpf. Furthermore, the relative fluorescence intensity, which corresponds to the level of apoptosis, was significantly increased ($p < 0.05$) in the whole body and the above-mentioned organs compared with the control (Figure 5B). ROS generation in the whole body of the embryo increased in a concentration-dependent manner (Figure 5B). Moreover, ROS levels in the eye, heart and tail regions were also
markedly increased in the embryos treated with 1 mg/L GO at 96 hpf (Figure 6A). In addition, GO down-regulated SOD activity in a concentration-dependent manner and markedly increased the concentration of MDA compared with the control (Figure S17) \( (p < 0.05) \).

**Genotoxicity**

The production of 8-OHdG in embryos increased with the GO concentration, and both 1 and 100 mg/L GO significantly increased the levels of 8-OHdG compared with the control \( (p < 0.05) \) (Figure S18A). However, the effects of GO on global DNA methylation levels were complex (Figure S14A). GO at 1 mg/L suppressed the level of global DNA methylation, while GO at 100 mg/L promoted methylation (Figure 18B).

**Discussion**

The chorion, a special biological substance that surrounds the embryo until 72 hpf in zebrafish, protects the embryo and serves as the first barrier against contact with nanoparticles (Cheng et al., 2007). We recently showed that graphene-based materials directly interact with amino groups on the surface of plant cells (Hu et al., 2014); however, for the chorion, this interaction is not clear. In the present study, we examined the interactions between GO and the chorion using optical microscopy, SEM and FT-IR spectra. Optical microscopy images revealed that GO adsorbed to the surface of chorions and embryos (Figure 2). A clear GO layer formed, which covered the protuberances and enveloped the chorion, resulting in the blockage of pore canals among the protuberances (Figure 2B). Some functional groups on the chorion were altered after the embryos were treated with 1 mg/L GO; for example, the number of \(-\text{OH}\) and \(\text{C} = \text{O}\) groups was increased (Figure S5), suggesting that GO adsorption on the chorion may occur due to interactions between hydroxyl groups.

The clogging of the chorion pore canals resulted in the isolation of the chorion from the culture medium and subsequently affected embryonic development (Cheng et al., 2007; Ong et al., 2014). In the present study, delayed hatching, cardiac dysfunction and malformation were observed in the embryos treated with GO. However, the negative effects that can be attributed to the isolation of embryos from the environment are not clear. Therefore, the oxygen concentration of the microenvironment in the chorion and damage to the embryos were measured. A hypoxic microenvironment was gradually formed in the embryos as the GO concentration increased, which was consistent with blockage of the pores on the surface of the chorion and the degree of envelopment of the chorions by GO (Figure 2). At 1 mg/L GO, the pores were unblocked, and the oxygen concentrations near the chorion and embryo were not significantly different from the control \( (p > 0.05) \). However, the level of blockage was increased and the oxygen concentration near the embryos was significantly decreased \( (p < 0.05) \) as the concentration of GO increased. Finally, the surface of the chorion was completely enveloped when the embryos were exposed to 100 mg/L GO, and both the area near the chorion and the embryo were markedly anoxic \( (p < 0.05) \), suggesting that the oxygen supply was insufficient for embryo development after complete GO envelopment. These data indicate that the level of hypoxia in the chorion increases as the concentration of GO increases in a dose-dependent manner (Figure 2). Furthermore, after the chorions

Figure 5. Effects of GO on apoptosis in embryos after 96 hpf. (A) Representative images of apoptosis in specific regions of the embryos. The arrows denote the primary apoptotic regions in the embryos. (B) Semiquantitative analysis of apoptosis in the whole body and specific regions of embryos at 96 hpf. The relative fluorescence intensity was adjusted by subtracting the auto-fluorescence of the embryos (Figure S16). Each experiment was conducted twice with three replicates and three fish per replicate. *\( p < 0.05 \). The scale bar is 20 \( \mu \text{m} \) in all images.
were removed, the oxygen concentrations in the solution containing embryos and GO were not significantly different from the control (Figure S6). These data indicate that the hypoxic microenvironment is mainly attributed to the envelopment of chorions by GO. It is interesting that the oxygen concentrations in chorions close to the embryos had a significant positive correlation with the spontaneous movement of embryos at up to 48 hpf ($R^2 = 0.84$) (Figure S13), which might have affected the subsequent hatching.

Previous studies have reported that zebrafish embryos displayed delayed hatching when exposed to 120 mg/L single-walled carbon nanotubes (SWCNTs) (Cheng et al., 2007) and 3 mg/L hydroxyapatite nanoparticles (Zhao et al., 2013). In contrast, SWCNTs did not affect the hatching of zebrafish embryos at 100 mg/L (Ong et al., 2014). These contradictions might be caused by differences in the physicochemical properties of nanomaterials and exposure level, such as their chemical composition, purity, size, surface charge, shape and structure. In the present work, a hatching delay was observed in embryos treated with 100 mg/L highly pure GO with 0.02% S and 0.01% K. Therefore, the negative effects on embryo development can mainly be attributed to the interactions of GO and organisms. Generally, delayed hatching might be caused by a change in the elasticity of the chorion, inhibition of hatching enzymes, hypoxia or the reduction of spontaneous movement (Cheng et al., 2007; Ong et al., 2014). GO sheets could exert mechanical force on the chorion surface, resulting in a change in the elasticity of the chorion (Figure 2B). Envelopment by GO also created a hypoxic microenvironment in the chorions (Figure 2C) and induced a hatching delay. Previous work illustrated that hypoxia-exposed sand snail (*Polinices sordidus*) and black bream (*Acanthopagrus butcheri*) embryos displayed delayed hatching compared with those exposed to normoxic treatments (Booth, 1995; Hassell et al., 2008). Furthermore, the spontaneous movement of embryos treated with 100 mg/L GO was markedly reduced compared with that of the control at 48 hpf (Figure 4C), which was another reason underlying the delay in embryo hatching. In addition, GO induced pericardial and yolk sac edema, which may have disturbed the activity of hatching enzymes (Figures 4D and S14) (Ong et al., 2014). Although 1 and 10 mg/L GO induced these malformations, GO at these concentrations did not affect the hatching of zebrafish embryos, suggesting that disturbance of...
hatching enzymes did not play a decisive role in the delayed hatching in the present study.

To examine the transport of GO into embryos, confocal microscopy was performed using GO-F. Given that the fluorescence intensity of GO-F is far greater than that of free FITC and that the fluorescence intensity increases linearly as a function of the concentration (Figure S8), the fluorescence signal in the embryo should be due to GO-F incorporation. This result revealed the limited capacity of the chorion to resist the invasion of GO, which was also supported by the results of paraffin sectioning, TEM and Raman spectra (Figure 3B–G). The mechanism by which GO permeates the chorion remains unclear. Silver nanoparticles (5–46 nm) were previously reported to cross the zebrafish chorion through pore canals via passive diffusion (Lee et al., 2007). The TEM images support the above hypothesis that GO spontaneously penetrates the chorion via passive diffusion because the chorion is an acellular envelope (Henn & Braunbeck, 2011). However, in contrast to Ag nanoparticles (NPs), GO penetrated the chorions via independent pore canals, as shown in Figure 3(B).

Previous research has shown that graphene or GO is internalized into human cells mainly via endocytosis with size-dependent uptake (Chowdhury et al., 2013; Li et al., 2013a; Linares et al., 2014). In the present work, vesicles containing GO were observed in embryos treated with 1 mg/L GO (Figure 3B3), which supports the above hypothesis and suggests that GO was taken up into zebrafish embryos via endocytosis. In this study, GO particles with diameters ranging from approximately 101 to 258 nm (mean of 147 nm) were suitable for uptake in cells (Win & Feng, 2005). However, accumulation of GO in embryos was not enhanced as the concentration of GO increased (Figure S9). This result disagrees with a report by Shang et al. (2014), who proposed that graphene quantum dots (GQDs) were taken up into human neural stem cells in a concentration- and time-dependent manner. Reduced accumulation in embryos exposed to high concentrations of GO may be attributed to the reduction of GO stability and the enhancement of aggregation in E3 solution, which contains inorganic ions (Na\(^{+}\), K\(^{+}\), Mg\(^{2+}\) and Ca\(^{2+}\)) (Chowdhury et al., 2013) that hinder penetration of the chorion. In this context, the chorion has the capacity to resist the invasion of GO at high concentrations. Using mathematical modeling, a recent study proposed that graphene microsheets enter cells via spontaneous membrane penetration at edge asperities and corners (Li et al., 2013b). Although the direct spontaneous membrane penetration of GO was not observed in this work, this pattern of GO penetration of embryos is possible as it penetrates the chorions, as presented in Figure 3(B1).

Once GO entered the embryo, it initially appeared around the embryo and selectively localized to the yolk sac, heart, eye and tail; additionally, it was also enriched in blood vessels, as shown in Figures 3(C) and S10. This agreed with a report stating that GO family nanomaterials primarily accumulated in the lungs, liver and spleen and were also enriched in blood vessels in mice (Lu et al., 2014). Many nanoparticles have been reported to affect the early development of zebrafish embryos, and malformations can result when the embryos are exposed to Ag NPs, Si NPs, CNTs and fullerenes (Bar-Ilan et al., 2009; Cheng & Cheng, 2012; Duan et al., 2013; Usenko et al., 2007). In the present study, three typical types of malformations, including pericardial/yolk sac edema, tail flexure and eye/head malformation (Figure S14) were observed, which were consistent with the localization of GO in embryos. After the chorions were removed, these three typical malformations were also observed in the embryos treated with 1 mg/L GO, suggesting that GO could induce malformation during zebrafish embryogenesis. Moreover, the incidence of tail/spinal cord flexure, pericardial/yolk sac edema and eye/head malformation increased depending on the concentration of GO; this was particularly true for pericardial/yolk sac edema. However, the accumulation of GO in embryos was not enhanced as the concentration of GO increased, as discussed above. The oxygen concentration in the chorion decreased as the GO concentration increased, which indicated that hypoxia enhanced the toxicity of GO. These results also suggest that the malformations induced by hypoxia are selective, and more malformations are typically observed in the pericardial/yolk sac (edema) and head, which may be because the heart and brain are more sensitive to hypoxia than other organs. Interestingly, the incidence of tail/spinal cord flexure initially increased and later decreased with increasing GO concentrations. These unique results differed from previous results obtained with cadmium and TiO\(_2\) nanoparticles, which continuously enhanced tail/spinal cord flexure as their concentrations increased (Clemente et al., 2014; Ladhar et al., 2014). Similarly, heart rates were significantly reduced in embryos exposed to 1 mg/L GO compared with control embryos; however, they were increased in the embryos exposed to 100 mg/L GO compared with control embryos. To increase the supply of oxygen under more severe hypoxic stress, the heart rates increased in the embryos treated with 100 mg/L GO. This phenomenon was not observed with Ag nanoparticles or nanophosphor particles, which steadily reduced the heart rate as their concentrations increased (George et al., 2014).

Apoptosis is considered as a general adverse effect of nanomaterials and is associated with various diseases and toxicities (Holden et al., 2013). Oxidative stress, including ROS generation, is the main adverse effect associated with GO and is regarded as the most likely mechanism of cell death (Lammel & Navas, 2014; Russier et al., 2013). The identification of apoptosis and ROS in specific organs is critical for interpreting and understanding the relevant diseases or adverse effects, such as heart rate abnormalities and malformations (Zhang et al., 2013). In the present study, both ROS and apoptosis were increased in the specific regions where GO was localized, suggesting that GO accumulation may result in oxidative stress and apoptosis during zebrafish embryogenesis.

Graphene is able to induce ROS generation and subsequently trigger apoptosis by activating the mitochondrial pathway (Li et al., 2012), which supports our result that apoptosis increased along with ROS generation and damage to the mitochondria (Figures 3 and 6). Carbon nanotubes (CNTs) might cause ROS generation and toxic effects in vivo or in vitro mainly due to the presence of surface defects (Yan et al., 2013). Similarly, structural defects are also considered to play a major role in the toxicity of graphene family materials (Xu et al., 2013), largely because of the high level of activity of guest atoms or molecules (Yan et al., 2013). Furthermore, in the present study, GO was observed to have sharp dentate edges (Figure 1C); thus, GO may damage the mitochondrial membrane and induce the generation of ROS via a direct physical process. It has been reported that these sharp edges can directly rupture the membranes of cells, bacteria and viruses, thereby inducing physical damage to organisms (Akhavan & Ghaderi, 2010; Liu et al., 2011). In addition, hypoxic environments also may induce excessive generation of ROS and apoptosis as well as alter energy metabolism and self-renewal in animal and human embryos (Forristal et al., 2013). These results indicate that there are many ways to generate ROS, cause apoptosis and induce malformations during embryogenesis. However, the generation of ROS is closely related to mitochondrial damage, largely because the mitochondria are the major ROS-producing organelle in zebrafish embryos.

The excessive generation of ROS significantly inhibited the activity of SOD, increased the MDA content, and caused oxidative stress. Previous studies have shown that oxidative...
damage to guanines in nascent DNA strands inhibits DNA methylation and reduces the level of DNA methylation (Turk et al., 1995). In this study, the global DNA methylation level in the embryos treated with 1 mg/L GO was significantly reduced, and the level of 8-OHdG adducts was increased, as presented in Figure S18. The decrease in DNA methylation might alter gene expression, which may result in developmental abnormalities, such as malformation and apoptosis (Fu et al., 2014; McGee et al., 2012). Therefore, the abnormal DNA methylation pattern induced by GO exposure may be the underlying mechanism of GO toxicity to embryos. GO at 100 mg/L caused an increase in the levels of both 8-OHdG adducts and DNA methylation, implying that the mechanism underlying the toxicity of GO to embryos is complex.

Conclusions

GO specifically enveloped and permeated the chorion, causing hypoxia in the chorion and toxicity during zebrafish embryogenesis. The enveloping effect directly resulted in the development of a markedly anoxic microenvironment near the embryo, and the GO exerted mechanical force on the surface of the chorion. At the same time, the spontaneous movement of the embryo was weakened, which was also attributed to the envelopment by GO. These adverse effects eventually led to delayed hatching during the development of zebrafish embryos. GO induced ROS, apoptosis, pericardial/yolk sac edema and tail/spinal cord flexure in a concentration-dependent manner. After entering into embryos, GO selectively localized to the yolk sac, heart, eye and tail. GO damaged mitochondria, disturbed antioxidant enzyme activity, induced ROS production and lipid peroxidation, and caused apoptosis and malformations in these regions. In addition, GO induced the formation of 8-OHdG adducts and altered DNA methylation. This work explored the specific adverse effects of GO on embryogenesis and highlighted the potential ecological and health risks of GO.

Declaration of interest

The authors declare no competing financial interests.

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References

Ahmed F, Rodrigues DF. 2013. Investigation of acute effects of graphene oxide on wastewater microbial community: a case study. J Hazard Mater 256:33–9.

Akhan O, Ghaederi E. 2010. Toxicity of graphene and graphene oxide nanowalls against bacteria. ACS Nano 4:5731–6.

Akhan O, Ghaederi E, Esfandiar A. 2011. Wrapping bacteria by graphene nanosheets for isolation from environment, reactivation by sonication, and inactivation by near-infrared irradiation. J Phys Chem B 115:6279–88.

Ashkarran AA, Ghavami M, Aghaverdi H, Stroeve P, Mahmoudi M. 2012. Bacterial effects and protein corona evaluations: crucial ignored factors in the prediction of bio-efficacy of various forms of silver nanoparticles. Chem Res Toxicol 25:1231–42.

Bar-Ilan O, Albrecht RM, Fako VE, Furgeson DY. 2009. toxicity assessments of multi sized gold and silver nanoparticles in zebrafish embryos. Small 5:1897–910.

Booth DT. 1995. Oxygen availability and embryonic development in sand snail (Polinices sordidus) egg masses. J Exp Biol 198:241–7.

Chen PJ, Wu WL, Wu KCW. 2013. The zerovalent iron nanoparticle causes higher developmental toxicity than its oxidation products in early life stages of medaka fish. Water Res 47:3899–909.

Cheng J, Cheng SH. 2012. Influence of carbon nanotube length on toxicity to zebrafish embryos. Int J Nanomed 7:3731–9.

Cheng J, Flahaut E, Cheng SH. 2007. Effect of carbon nanotubes on developing zebrafish (Danio rerio) embryos. Environ Toxicol Chem 26:708–16.

Chio CP, Chen WY, Chou WC, Hsieh NF, Ling MP, Liao CM. 2012. Assessing the potential risks to zebrafish posed by environmentally relevant copper and silver nanoparticles. Sci Total Environ 420:11–18.

Choi SWY, Cheng SH, Yu KN. 2010. Radioadaptive response induced by alpha-particle-induced stress communicated in vivo between zebrafish embryos. Environ Sci Technol 44:8829–34.

Chowdhury I, Duch, MC, Mansukhani ND, Hersam MC, Bouchard D. 2013. Colloidal properties and stability of graphene oxide nanomaterials in the aquatic environment. Environ Sci Technol 47:6288–96.

Clemente Z, Castro VLSS, Moura MAM, Jonsson CM, Faceto LF. 2014. Toxicity assessment of TiO2 nanoparticles in zebrafish embryos under different exposure conditions. Aquat Toxicol 147:129–39.

Dikin DA, Stankovich S, Zimney EJ, Piner RD, Dommett GHB, Evmenenko G, et al. 2007. Preparation and characterization of graphene oxide paper. Nature 448:457–60.

Duan JC, Yu YB, Li Y, Yu Y, Sun ZW. 2013. Cardiovascular toxicity evaluation of silica nanoparticles in endothelial cells and zebrafish model. Biomaterials 34:5853–62.

Fako VE, Furgeson DY. 2009. Zebrafish as a correlative and predictive model for assessing biomaterial nanotoxicity. Adv Drug Deliv Rev 61:478–86.

Ferris CE, Christensen DR, Chimney FE, Petruzelli R, Parry KL, Sanchez-Elsner T, et al. 2013. Environmental oxygen tension regulates the energy metabolism and self-renewal of human embryonic stem cells. PLoS One 8:e62507.

Fu MZ, Wu XY, He J, Zhang Y, Hua S. 2014. Natrium fluoride influences methylation modifications and induces apoptosis in mouse early embryos. Energy Environ Sci 48:10398–405.

George S, Gardner H, Seng EK, Chang H, Wang C, Fang CHY, et al. 2014. Differential effect of solar light in increasing the toxicity of silver and titanium dioxide nanoparticles to a fish cell line and zebrafish embryos. Environ Sci Technol 48:6374–82.

Gollavelli G, Ling YC. 2012. Multi-functional graphene as an in vitro and in vivo imaging probe. Biomaterials 33:3532–45.

Hassell KL, Coutin PC, Noguegoda D. 2008. Hypoxia impairs embryo development and survival in black bream (Acanthopagrus butcheri). Mar Pollut Bul 57:302–6.

Heiden TCK, Dengler E, Kao WJ, Heideman W, Peterson RE. 2007. Developmental toxicity of low generation PAMAM dendrimers in zebrafish. Toxicol Appl Pharmacol 250:70–9.

Henn K, Braunbeck T. 2011. Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). Comp Biochem Phys C 153:91–9.

Holden PA, Nisbet RM, Lenihan HS, Miller RJ, Cherr GN, Schimel JP, et al. 2013. Ecological nanotoxicology: integrating nanomaterial hazard considerations across the subcellular, population, community, and ecosystems levels. Accounts Chem Res 46:813–22.

Hu W, Peng C, Ly M, Li X, Zhang Y, Chen N, et al. 2011. Protein corona-mediated mitigation of cytotoxicity of graphene oxide. ACS Nano 5:3693–700.

Hu XG, Kang J, Lu KC, Zhou RR, Mu L, Zhou QX. 2014. Graphene oxide amplifies the phytotoxicity of arsenic in wheat. Sci Rep 4:6122.

Hu XG, Zhou QX. 2013. Health and ecosystem risks of graphene. Chem Rev 113:3815–35.

Huang LJ, Wang YX, Huang Z, Tang JG, Wang Y, Liu JX, et al. 2014. Effects of graphene/silver nanohybrid additives on electrochemical properties of magnesium-based amorphous alloy. J Power Sources 269:716–22.

Ilan OB, Chuang CC, Schwahn DJ, Yang S, Joshi S, Pedersen JA, et al. 2013. TiO2 Nanoparticle exposure and illumination during zebrafish development: mortality at parts per billion concentrations. Environ Sci Technol 47:4726–33.

Ladhar C, Geoffroy B, Cambier S, Treguer-Delapierre M, Durand E, Brethes D, et al. 2014. Impact of dietary cadmium sulphide nanoparticles on Danio rerio zebrafish at very low contamination pressure. Nanotoxicology 8:676–85.
Lammel T, Navas JM. 2014. Graphene nanoplatelets spontaneously translocate into the cytosol and physically interact with cellular organelles in the fish cell line LHC-1. Aquat Toxicol 150: 55–65.

Lee KJ, Nallathamby PD, Browning LM, Osgood CJ, Xu XHN. 2007. In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. ACS Nano 1: 133–43.

Li B, Yang J, Huang Q, Zhang Y, Peng C, Zhang Y, et al. 2013a. Biodistribution and pulmonary toxicity of intratracheally instilled graphene oxide in mice. NPG Asia Mater 5:1–8.

Li Y, Liu Y, Fu YJ, Wei TT, Le Guyader L, Gao G, et al. 2012. The triggering of apoptosis in macrophages by pristine graphene through the MAPK and TGF-beta signaling pathways. Biomaterials 33:402–11.

Li YF, Yuan HY, von dem Bussche A, Creighton M, Hurt RH, Kane AB, et al. 2013b. Graphene microsheets enter cells through spontaneous membrane penetration at edge asperities and corner sites. Proc Natl Acad Sci USA 110:12295–300.

Linares J, Matesanz MC, Vila M, Feito MJ, González G, Vallet-Regí M, et al. 2014. Endocytic mechanisms of graphene oxide nanosheets in osteoblasts, hepatocytes and macrophages. ACS Appl Mater Inter 6: 13697–706.

Liu SB, Zeng TH, Hofmann M, Burcombe E, Wei J, Jiang RR, et al. 2011. Antibacterial activity of graphite, graphite oxide, graphene oxide, and reduced graphene oxide: membrane and oxidative stress. ACS Nano 5: 6971–980.

Lu YJ, Lin CW, Yang HW, Lin KJ, Wey SP, Sun CL, et al. 2014. Biodistribution of PEGylated graphene oxide nanoribbons and their application in cancer chemo-photothermal therapy. Carbon 74: 83–95.

Mahmoudi M, Akhavan O, Ghavami M, Rezaee F, Ghiasi SMA. 2012. Graphene oxide strongly inhibits amyloid beta fibrillation. Nanoscale 4:7322–5.

McGee SP, Cooper EM, Stapleton HM, Volz DC. 2012. Early zebrafish embryogenesis is susceptible to developmental TDCPP exposure. Environ Health Perspect 120:1585–91.

Ong KJ, Zhao X, Thistle ME, MacCormack TJ, Clark RJ, Ma G, et al. 2014. Mechanistic insights into the effect of nanoparticle s on zebrafish hatch. Nanotoxicology 8:295–304.

Peralta-Videa JR, Zhao L, Lopez-Moreno ML, Rosa G, Hong J, Gardea-Torresdey JL. 2011. Nanomaterials and the environment: a review for the biennium 2008-10. J Hazard Mater 186:1–15.

Ramsahoye BH. 2002. Measurement of genome wide DNA methylation by reversed-phase high-performance lipid chromatography. Methods 27:156–61.

Russier J, Treossi E, Scarisi A, Perrozzi F, Dumortier H, Ottaviano L, et al. 2013. Evidencing the mask effect of graphene oxide: a comparative study on primary human and murine phagocytic cells. Nanoscale 5:11234–47.

Sanchez VC, Jachak A, Hurt RH, Kane AB. 2012. Biological interactions of graphene-family nanomaterials: an interdisciplinary review. Chem Res Toxicol 25:15–34.

Schwab F, Bucheli TD, Lukhele LP, Magrez A, Nowack B, Sigg L, et al. 2011. Are carbon nanotube effects on green algae caused by shading and agglomeration? Environ Sci Technol 45:6136–44.

Serag MF, Kaji N, Guillard C, Okamoto Y, Terasaka K, Jabasini M, et al. 2011. Trafficking and subcellular localization of multiwalled carbon nanotubes in plant cells. ACS Nano 5:493–9.

Shang WH, Zhang XY, Zhang M, Fan ZT, Sun, YT, Han M, et al. 2014. The uptake mechanism and biocompatibility of graphene quantum dots with human neural stem cells. Nanoscale 6:5799–806.

Shi X, Chang H, Chen S, Lai C, Khademhosseini A, Wu H. 2012. Regulating cellular behavior on few-layer reduced graphene oxide films with well-controlled reduction states. Adv Funct Mater 22: 751–9.

Su Y, Hu M, Fan C, He Y, Li Q, Li W, et al. 2010. The cytotoxicity of CdTe quantum dots and the relative contributions from released cadmium ions and nanoparticle properties. Biomaterials 31:4829–34.

Turk PW, Laayoun A, Smith SS, Weitzman SA. 1995. DNA adduct 8-hydroxy-2′-deoxyguanosine (8-hydroxyguanine) affects function of human DNA methyltransferase. Carcinogenesis 16:1253–5.

Usenko CY, Harper SL, Tanguay RL. 2007. In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. Carbon 45:1891–8.

Wahid MH, Eroglu E, Chen X, Smith SM, Raston CL. 2013. Entrapment of Chlorella vulgaris cells within graphene oxide layers. ACS Adv 3: 8180–3.

Wecinski PN, Metz KM, Heiden TCK, Louis KM, Mangham AN, Hamers RJ, et al. 2013. Toxicity of oxidatively degraded quantum dots to developing zebrafish (Danio rerio). Environ Sci Techno 47:9132–9.

Win KY, Feng SS. 2005. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. Biomaterials 26:2713–22.

Wu M, Kempaiah R, Huang PJ, Maheshwari V, Liu JW. 2011. Adsorption and desorption of DNA on graphene oxide studied by fluorescently labeled oligonucleotides. Langmuir 27:2731–8.

Xu MS, Liang T, Shi MM, Chen HZ. 2013. Graphene-like two-dimensional materials. Chem Rev 113:3766–98.

Yan L, Gu ZJ, Zhao YL. 2013. Chemical mechanisms of the toxicological properties of nanomaterials: generation of intracellular reactive oxygen species. Chem-Asian J 8:2342–53.

Zhang H, Peng C, Yang J, Lv M, Liu R, He D, et al. 2013. Uniform ultrasmall graphene oxide nanosheets with low cytotoxicity and high cellular uptake. ACS Appl Mater Inter 5:1761–7.

Zhao XX, Ong KJ, Ede JD, Stafford JL, Ng KW, Goss GG, et al. 2013. Evaluating the toxicity of hydroxyapatite nanoparticles in catfish cells and zebrafish embryos. Small 9:1734–41.

Zhou Q, Kong F, Zhu L. 2004. An introduction to ecotoxicology (in Chinese). Beijing, China: Science Press.

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