Supporting Information

Specific nanotoxicity of graphene oxide during zebrafish embryogenesis

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Zebrafish maintenance

For all of the experiments in this study, zebrafish embryos (AB strain) were maintained at 26-30 °C in a buffered solution consisting of 60 mg/L Instant Ocean salts in reverse osmosis-purified water under a 14 h/10 h light/dark cycle. The zebrafish were fed brine shrimp twice daily.

Exposure of embryos to GO

Embryos were collected immediately after fertilization and sorted to remove feces and infertile eggs. The collected embryos were transferred to an incubator at a temperature of 27 ± 1 °C until 2 hours post-fertilization (hpf). Next, healthy embryos were selected using a stereomicroscope (Olympus, SZX16, Japan) and were transferred to 96-well plates (1 embryo per well containing medium, 20 embryos per group), suspended in 0.2 mL of E3 medium (5 mM NaCl, 0.33 mM CaCl₂, 0.17 mM KCl, 0.33 mM MgSO₄, pH 7.4) and treated with GO at concentrations ranging from 0.01 to 100 mg/L.

Mortality, hatching, heart rate, spontaneous movement and malformation

Larval emergence from the egg chorion was considered successful hatching. The embryos were transferred to 0.016 M tricaine for anesthetization. Then, the embryos and larvae were imaged to examine their structure, and the incidences of typical tail flexure, cardiac/yolk sac edema and eye/head defects were recorded. The heart rate and spontaneous movement were assessed by continuous observation for 10 min at room temperature.

Tracing the translocation of GO *in vivo*

The fluorescence intensity of hybridized GO-FITC (GO-F) was detected using a fluorescence spectrometer (LS 55, Perkin Elmer). A 1-mL aliquot of GO-F solution was added to a 35-mm-diameter confocal dish containing three embryos developing beyond 24 hpf. Next, real-time monitoring of GO-F transport in embryos was performed via confocal microscopy (Leica, TCS SP5, Germany).

Scanning electron microscopy (SEM)

Embryos older than 24 hpf were fixed using 2.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.2) for 4 h at room temperature, subsequently washed with 30%, 50%, 80%, 90% and 100% (twice) ethanol and lyophilized prior to SEM imaging.

Hypoxic microenvironment of the embryos

Prior to use, the sensor was polarized overnight at -0.80 V to remove oxygen. Then, six embryos incubated for 48 h in E3 solution containing 0, 0.01, 0.1, 1, 10 or 100 mg/L GO were fixed using low-melting-point agarose (1%). Hypoxia in the microenvironment was detected using an oxygen microsensor. A CAL300 calibration chamber was used to calibrate the Profix 3105 software, and the samples were detected at 50- μ m intervals. The depth of analysis ranged from 0 to 250 μ m, which is consistent with the interspace between the chorion and the embryonic matter. The sensor was held at each sampling site for 5 s. To differentiate the contributions of GO envelopment and mitochondrial respiration to the hypoxic microenvironment, the chorions from embryos at 2.5 hpf were softened by incubation in 2 mg/mL Pronase E

(Sigma-Aldrich) for 1 min at 28.0 °C, followed by mechanical removal. Twenty dechorionated embryos were incubated in six-well plates and exposed 0 or 1 mg/L GO. At 48 hpf, the oxygen concentration in the solution was measured using an oxygen microsensor.

Microstructure observation

Zebrafish were fixed in picric acid for 12 h at 4 °C, rinsed for 24 h using distilled water, and dehydrated using a graded ethanol series. The tissues were subsequently embedded in paraffin and sectioned at 7 µm using a Leica EG1150H module, and imaged via optical microscopy (Olympus X71; Olympus, Tokyo, Japan).

Transmission electron microscopy (TEM)

Zebrafish were fixed in 2.5% glutaraldehyde overnight at 4°C, and post fixed in 1% osmium tetroxide for 2 h at 25°C, dehydrated in a graded ethanol and propylene oxide series, and embedded in Spurr's epoxy resin. Samples were sectioned (60 – 80 nm) using a Leica EM UC7 ultramicrotome, collected onto Pioloform-coated grid slots, and imaged via TEM (Hitachi HT7700, Tokyo, Japan).

Apoptosis and ROS measurements

Briefly, embryos were stained in culture medium containing the vital dye 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (10 µM) or the vital dye acridine orange (AO) (2 µg/mL) for 1 h and then thoroughly washed twice in culture medium alone. Subsequently, the embryos were transferred to 0.016 M tricaine for anesthetization. The eye, heart and tail regions of each embryo were imaged using a fluorescence microscope (Olympus X71; Olympus, Tokyo, Japan). The relative

fluorescence intensity was quantified using Olympus FV-10 ASW 3.1 software.

Determination of DNA methylation

After DNA was isolated from the head and heart regions of zebrafish larvae, approximately 50 μ L of extracted DNA was hydrolyzed in 25 μ L of 70% perchloric acid at 80 °C for 5 h. The pH was adjusted to between 3 and 5 using 2 M KOH, and the sample was allowed to precipitate overnight at –20 °C. DNA methylation was measured via HPLC. The mobile phase was a mixed liquor containing 10% methanol, 5 mM pentane-1-sulfonic acid sodium salt and 0.2% triethylamine. Nucleoside standards were prepared in deionized H₂O and stored at –20 °C until their use in HPLC analysis.

Determination of 8-hydroxy-2-deoxyguanosine (8-OHdG) levels

For this assay, 30 fish were used, and two replicates were performed (60 fish per treatment). Embryos were exposed to E3 solution or GO (0.01, 1 and 100 mg/L) until 96 hpf. Then, the fish were collected, flash frozen, and stored at –80 °C until analysis. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) and quantified using a T90 spectrophotometer (Purkinje General, Beijing). The production of 8-OHdG was determined using a Synergy 4 microplate reader (Bio Tek, USA) to read the absorbance at $\lambda = 405$ nm. The concentrations of 8-OHdG were normalized to the mass of DNA.

Figure S1. UV-vis absorption spectrum of 100 mg/L GO in E3 solution for 7 d. (A) Initial GO in E3 solution. (B) GO in E3 solution for 7 d.

Figure S2. Energy-dispersive spectra of graphene oxide.

Figure S3. Adsorption of GO to zebrafish embryos older than 96 hpf.

Figure S4. Transmission electron microscopy images of the zebrafish chorion.

Figure S5. FT-IR spectra display the interactions of the chorion with GO. Embryos

were incubated in E3 solution with or without 1 mg/L GO and developed for 36 h.

Figure S6. The oxygen concentrations of solution-incubated dechorionated embryos. Twenty embryos were used per treatment, and the treatments were performed in triplicate.

Figure S7. Localization of GO in zebrafish embryos at 24 hpf. The red arrows indicate GO. Each experiment was conducted twice with 2 replicates and 3 fish per replicate.

Figure S8. The fluorescence intensity of FITC-labeled GO. The linear relationship between fluorescence intensity and the concentration of FITC-labeled GO (A). The fluorescence intensity of GO-F and F (B). F, fluorescein isothiocyanate (FITC).

Figure S9. Uptake of GO in the chest of embryos at 96 hpf. Semiquantitative analysis of GO in embryos was performed by determining the ratio of the GO aggregate area to the area of the whole image using the software program Image J.

Figure S10. Localization of GO in a representative paraffin section at 96 hpf. The red arrows indicate the primary areas in which GO localized. Each experiment was conducted twice with 2 replicates and 3 fish per replicate.

Figure S11. Positions of the zebrafish sections made using the ultramicrotome.

Figure S12. Mortality of zebrafish embryos exposed to GO. The mortality of embryos increased in a dose- and time-dependent manner. The data are expressed as the means $\pm$ standard deviation (SD) of three independent experiments. In each experiment, 40 embryos were used. *, $p < 0.05$.

Figure S13. The relationships between the oxygen concentrations in the chorions near the embryos and spontaneous movement of the embryos at 48 hpf.

Figure S14. Malformations of embryos older than 96 hpf exposed to GO. Optical microscopy images of deformed (A - J) and normal (K) embryos. Tail/spinal cord flexure (A - D); pericardial/yolk sac edema (E-F); pericardial/yolk sac edema and tail/spinal cord flexure (G); eye/head malformation (H - I); and a dead embryo (J). The arrows indicate the respective malformations in the embryos. The scale bar is 450 μm in all images. ($n = 40$)

Figure S15. Effects of GO on the incidence of malformations in the embryos from which the chorion was removed. The chorion was removed using 2 mg/mL protease E

at 2.5 hpf, and the incidence of malformations was determined at 96 hpf. Each experiment was conducted 3 times with $n = 20$ replicates. *, $p < 0.05$ compared with the control.

Figure S16. Representative image of the auto-fluorescence of embryos at 96 hpf (excitation at 488 nm).

Figure S17. Oxidative stress induced by GO during zebrafish embryogenesis. (A) GO inhibits the activity of SOD. (B) GO enhances the MDA content in zebrafish embryos at 96 hpf. Each experiment was conducted twice with 2 replicates and 30 fish per replicate. Differences were considered significant at $p < 0.05$.

Figure S18. Genotoxicity of GO in embryos at 96 hpf. (A) Oxidative DNA damage, expressed as the concentration of 8-hydroxy-2-deoxyguanosine (pg/mL) normalized to the DNA concentration ($\mu\text{g/mL}$), in fish exposed to 0, 0.01, 1 or 100 mg/L GO until 96 hpf. (B) The effects of GO on global DNA methylation in zebrafish. Each experiment was conducted twice with 2 replicates and 30 fish per replicate. Differences were considered significant at $p < 0.05$.

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