Original Article

Toxicity of Multi-Walled Carbon Nanotubes, Graphene Oxide, and Reduced Graphene Oxide to Zebrafish Embryos*

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Abstract

Objective This study was aimed to investigate the toxic effects of 3 nanomaterials, i.e. multi-walled carbon nanotubes (MWCNTs), graphene oxide (GO), and reduced graphene oxide (RGO), on zebrafish embryos.

Methods The 2-h post-fertilization (hpf) zebrafish embryos were exposed to MWCNTs, GO, and RGO at different concentrations (1, 5, 10, 50, 100 mg/L) for 96 h. Afterwards, the effects of the 3 nanomateria on spontaneous movement, heart rate, hatching rate, length of larvae, mortality, and malformations ls were evaluated.

Results Statistical analysis indicated that RGO significantly inhibited the hatching of zebrafish embryos. Furthermore, RGO and MWCNTs decreased the length of the hatched larvae at 96 hpf. No obvious morphological malformation or mortality was observed in the zebrafish embryos after exposure to the three nanomaterials.

Conclusion MWCNTs, GO, and RGO were all toxic to zebrafish embryos to influence embryos hatching and larvae length. Although no obvious morphological malformation and mortality were observed in exposed zebrafish embryos, further studies on the toxicity of the three nanomaterials are still needed.

Key words: Zebrafish Embryos; Toxicity; Multi-Walled Carbon Nanotubes; Graphene Oxide; Reduced Graphene Oxide

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INTRODUCTION

Recently, carbon nanomaterials have attracted considerable attention in different areas of nanotechnology research. And more studies have been conducted on graphene, a one-atom-thick monolayer of sp2-bonded carbon atoms arranged in a two-dimensional honeycomb structure[1], due to its unique optical, electrical, mechanical, and thermal properties. One of the major methods of preparing large amounts of graphene is reducing graphene oxide, and graphene prepared by this method is called reduced graphene oxide (RGO)[2]. Carbon nanotubes (CNTs), a cylinder made of graphene[3], have many properties similar to those of graphene. Graphene oxide (GO), the product of chemical exfoliation of graphite, is the most important

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derivative of graphene with the presence of oxygen functional groups such as carboxylates, epoxides, and hydroxyls at the basal planes and edges of graphene sheets[4]. Because of their unique and desirable characteristics, the application of nanomaterials, such as RGO, CNTs, and GO, have significantly increased in the fields of field-effect transistors, chemical sensors and biosensors, organic solar cells, and flexible displays[5-7]. With more application of nanomaterials in the production of goods used in our daily life, such as pharmaceutical, biomedical, cosmetic, and sporting products, it is almost unavoidable for them to release into the environment through air, water, and soil. So, besides the benefit from the use of nanomaterials products, it is very important to improve the people's awareness about toxicity of these nanomaterials to prevent their harmful environmental effects[8-9]. In addition to immediate effects, the potential toxicity to the environment after exposure to nanomaterials remains uncertain and close attention should be paid to it. Therefore, the study to determine nanotoxicity has great importance and high scientific, social, and economic value[10]. In recent years, some studies focused on the toxicity of metallic nanoparticles, semiconductor quantum dots, carbon materials and others[11-14]. However, the environmental risks of the novel carbon nanomaterials still remain unclear. Surface characteristics, different structural features, and nanoparticle aggregation in actual environment may change their toxicity[15]. In the recent years, a few studies reported the environmental effects of carbon nanomaterials. Zhang et al. reported some studies on the toxicological responses of carbon nanomaterials to different cell types and mice, and discovered that multi-walled carbon nanotubes (MWCNTs) was very important factor in cytotoxicit due to its surface hydrophilicity[16-22]. Zhu et al.[23] demonstrated that aggregation of buckminster- fullerene (nC_{60}) decreased the survival and hatching rates of zebrafish. Zhang et al.[24] and Liu et al.[25] reported the respective effect of graphene and GO on human health. Akhavan et al.[26] investigated the toxicity of graphene and GO against bacteria. In addition, the effect of graphene on terrestrial plants was also reported[27]. These carbon nanomaterials have a certain effect on human health, plants, animals, and so on. However, the toxicity of RGO, GO, and MWCNTs in aquatic environment remains unclear. As these nanomaterials have been widely studied and applied, their effect and the potential future impact on aquatic environment should not be ignored and the knowledge about their fundamental toxicity is needed. So, the present study aiming to evaluate developmental toxicity of the three carbon nanomaterials including MWCNTs, GO, and RGO in aquatic environment will provide useful information about the toxicity of these nanomaterials for the practical and safe applications in future.

Zebrafish, an aquatic vertebrate species, is used as a basic model organism for the assessment of toxicity in aquatic environment according to the reports of the National Institute of Environmental Health Sciences (NIEHS) and the Institute for Environment and Sustainability (IES)[28-29]. Zabrafish embryos is an alternative model for the test for evaluating developmental toxicity of chemicals during early life stage with the characteristics of small-scale, high throughput, and easy observations[30].

In this study, an embryo-larval test was performed by using 2 hpf embryos of zebrafish to investigate the toxicity of MWCNTs, GO, and RGO on early stages of development. The tests' concentrations ranged from 1 mg/L to 100 mg/L. And major endpoints such as spontaneous movement in 20 s, heart rate, hatching rate, length of larvae, mortality, and malformation were examined.

MATERIALS AND METHODS

Nanomaterials: MWCNTs, GO, and RGO

MWCNTs (outer diameter, 10-20 nm; length, 10-30 μm) were purchased from Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences. GO was synthesized from natural graphite using a modified Hummer's method[31]. Briefly, potassium permanganate ($KMnO_4$) (5 g) was slowly added to a suspension of graphite (1 g) and potassium nitrate (KNO_3) (5 g) in 98% sulfuric acid (H_2SO_4, 150 mL) while keeping the temperature under 10 °C. The mixture was heated to 40 °C in an oil bath for 10 h and then stirred at 90 °C for 2 h. Then, a 30% hydrogen peroxide (H_2O_2) solution was added dropwise to the mixture until gas evolution (bubbling) ceased. The mixture was heated to 70 °C in the oil bath for 2 h and then allowed to cool to room temperature. For purification, the resulting mixture was washed and centrifuged for several times, first with 1:1 (v/v) mixture of 20% H_2O_2 and 10% H_2SO_4 and then with deionized water. GO was

prepared after the ultrasonication. RGO was synthesized by using a hydrazine reduction process of GO[32]. The water-soluble GO (150 mL) was sonicated for 30 min and then reduced by using hydrazine monohydrate after the pH of this solution was adjusted to 10 with diluted ammonia. The product was centrifuged and washed repeatedly with distilled water. The concentration of GO and RGO was calculated by filtering 500 mL of the dispersion through a weighed filter paper, then drying in a vacuum oven overnight and measuring the mass deposited of GO or RGO[33].

Zebrafish Keeping and Embryo Collection

Wild-type zebrafish were purchased from a local commercial source (Gao Feng Aquarium, Beijng, China). The zebrafish were kept in the flow-through feeding equipment (made by Esen Corp.) at 26 °C with a 14-h light/10-h dark cycle and fed on bloodworms, dry flake food, and brine shrimp twice daily. Zebrafish embryos were obtained from spawning adults in groups of male and female zebrafish (ratio=2:1) in spawning boxes overnight. Spawning was induced in the morning when the light was turned on and the embryos were collected after 30 min. The collected zebrafish embryos were rinsed for 3 times with standard water[34]. The developmental stage of the embryos was determined according to the method described by Kimmel et al.[35]. Embryos were examined under a dissecting microscope to ensure that normal embryos and embryos at a certain blastula stage were selected for the exposure experiments.

Embryo Toxicity Test

Twenty embryos were selected and exposed to MWCNTs, GO, and RGO at 6 concentrations (0, 1, 5, 10, 50, and 100 mg/L) dispersed in standard water. Subsequently, the embryos were transferred into 24-well multiplates with 1 embryo per well. The concentrations were set on the basis of previous studies[25,36] and feasibility of the tests. Three replicates were used for each concentration. The solutions were changed once per day. All 24-well multiplates were kept at 28±0.5 °C with a 14-h light/10-h dark cycle.

The development of zebrafish embryos and larvae was observed at specified times with microscope (OLYMPUS, CX21BIM). Furthermore, the toxicological endpoint corresponding to the embryonic developmental stages were examined at 8-96 hpf, including spontaneous movement in 20 s, heart rate, hatching rate, length of larvae, mortality, and malformations. The heart beat was observed clearly and recorded by an experimenter with a stopwatch. The hatched zebrafish embryos were also observed with microscope. The length of larvae was measured with Aigo GE-5 (made by Aigo Corp). For mortality, the death was defined as lethal toxicological endpoints proposed by Nagel[37] for zebrafish embryos and absence of heartbeat for larvae[38] under the microscope. Malformations were observed under the microscope as well. In these tests, 5 zebrafish embryos and larvae were randomly selected for recording from each replicate.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA). Data were shown as the mean±standard error and evaluated by using one-way analysis of variance (ANOVA) with post-hoc least significant difference (LSD) comparisons of means. The differences were considered significant for $P<0.05$.

RESULTS

The Characterizations of MWCNTs, GO, and RGO

The Scanning Electron Microscopy (SEM) images of MWCNs, GO, and RGO are shown in Figure 1a-c. The surface of MWCNTs was clearly observed. The diameters of MWCNTs ranged from 10 nm to 20 nm, which was consistent with the data provided by the manufacturer. In Figure 1b and c, many flakes with single and few layers were observed in GO and RGO.

Figure 1d shows the FT-IR spectra of MWCNTs, GO, and RGO. For MWCNTs, no obvious functional groups were observed. For GO, the band at 3400 cm^{-1} was attributed to -OH stretching, the vibration of C-O of carboxylic acid was at 1380 cm^{-1}. The peaks at 1729 cm^{-1} and 1620 cm^{-1} were attributed to C=O stretching vibration, and the peak at 1065 cm^{-1} was suggested to vibration of C-O (alkoxy). In the FT-IR spectra of RGO (Figure 1d, the third line), the band at 3400 cm^{-1} and 1620 cm^{-1} indicated a small amount of unreduced-OH and C=O groups. No obvious carbonyl peak, C-O-C peak, or epoxy group signals were observed.

The Raman spectra of MWCNTs, GO, and RGO is shown in Figure 4e. A D peak around 1350 cm^{-1} and a G peak around 1583 cm^{-1} were observed for the MWCNTs sample. For GO, a D peak around 1350 cm^{-1} and a G peak around 1596 cm^{-1} were observed. After

reduction, the D peak remained, while the G peak shifted to 1590 cm^{-1}, confirming the reduction of GO to RGO. And this was also resulted in a higher I_D/I_G ratio because of a decrease in the sp^2 cluster size for the removing of oxygen groups, which was consistent with previous findings[39].

The dispersity of MWCNTs, GO, and RGO was obtained by zeta-potential and size distribution measurements. In Table 1, the zeta-potentials of MWCNTs, GO, and RGO were -24.7±0.8, -56.7±1.5 and -25.4±1.4 mV, respectively. And the size distributions of these three nanomaterials were 277.7±37.8, 511.8±25.7, and 391.2±72.0 nm, respectively.

The Agglomerates on Zebrafish Chorion

After 24 hpf zebrafish embryos were exposed to the 3 nanomaterials, they adhered to the surface of the chorion of the zebrafish, and the aggregation of the nanoparticles at the surface of the chorion increased with the increase in the concentrations of the 3 nanomaterials (Figure 2).

Table 1. Zeta-potential and Size Distribution of MWCNTs, GO, and RGO

Items	Zeta-potential (mV)	Size distribution (nm)
MWCNTs	-24.7±0.8	277.7±37.8
GO	-56.7±1.5	511.8±25.7
RGO	-25.4±1.4	391.2±72.0

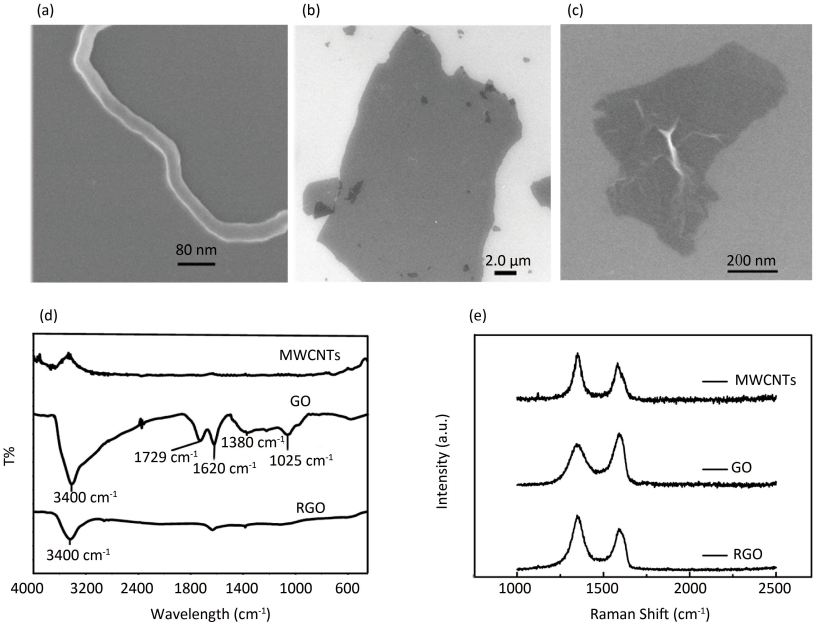

Figure 1. SEM images of (a) MWCNTs, (b) GO, and (c) RGO. (d) FT-IR spectra of MWCNTs, GO, and RGO. (e) Raman spectra of MWCNTs, GO, and RGO.

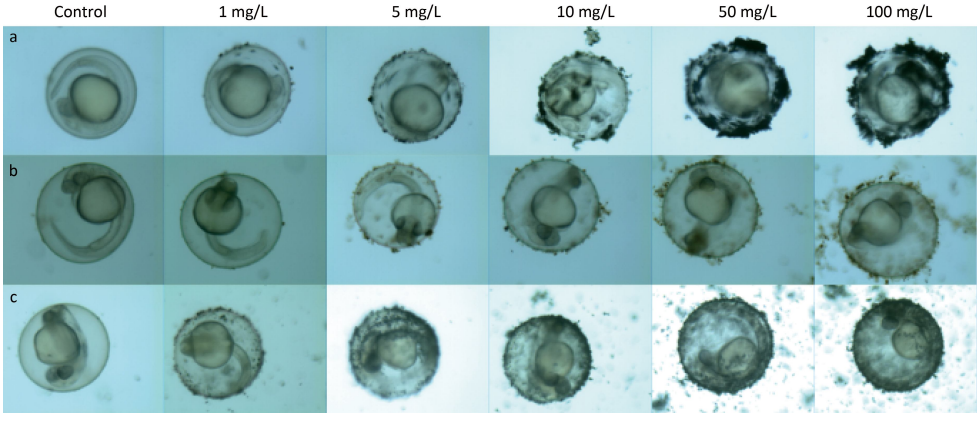

Figure 2. Effect of exposure to different concentrations (control, 1, 5, 10, 50, and 100 mg/L) of (a) MWCNTs, (b) GO, and (c) RGO for 24 h on the surface of the chorion of zebrafish embryos.

Spontaneous Movement in 20 s

Spontaneous movement in 20 s of zebrafish embryos was examined at 24 hpf. In Figure 3, no significant effect (P<0.05) was observed on spontaneous movement of zebrafish embryos exposed to suspensions of MWCNTs, GO, and RGO, which indicated that the development of zebrafish embryos treated with MWCNTs, GO, or RGO (1-100 mg/L) at 24 hpf was unaffected.

Heart Rate

The heart rates of zebrafish embryos at 48 hpf and larvae at 96 hpf were recorded after exposure to the 3 nanomaterials (MWCNTs, GO, and RGO) at different concentrations (0, 1, 5, 10, 50, and 100 mg/L). The results are shown in Figure 4; the heart rates of embryos exposed to RGO (1-100 mg/L) at 48 hpf and larvae at 96 hpf were similar to those of the control group (0 mg/L). For embryos treated with MWCNTs, the heart beats of larvae at 96 hpf was significantly (P<0.05) reduced from 5 to 100 mg/L, while no obvious change was observed in the treatments at 48 hpf. The heart rate of embryos treated with GO was significantly (P<0.05) decreased at 100 mg/L at 48 hpf and dropped from 5 to 100 mg/L at 96 hpf. These results and comparison of the effect of the 3 nanomaterials on the heart rate of zebrafish embryos indicated that MWCNTs and GO had effects on the heart rate, while RGO had no such effect.

Hatching Success

Hatching between 48 and 72 hpf is considered as a critical stage for embryogenesis of zebrafish, and zebrafish embryos hatched from the chorion[40]. The hatching rates of zebrafish embryos exposed to the 3 nanomaterials are shown in Figure 5. Compared with the control group, the RGO-treated group showed a significant dose-dependent decrease in the hatching rate (P<0.05). The hatching rates for embryos treated with RGO were 75.9%, 71.4%, 28.1%, and 25.8% at 5, 10, 50, and 100 mg/L, respectively. On the other hand, MWCNTs or GO had no effect on the hatching. These results indicated that RGO induced developmental delay and significant toxicity (P<0.05) to zebrafish embryos.

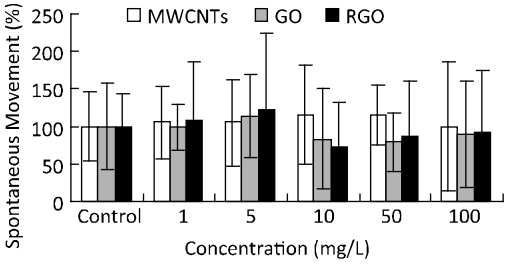

Figure 3. Spontaneous movement in 20 s of zebrafish embryos at 24 hpf exposed to MWCNTs, GO, and RGO at concentrations from 1 to 100 mg/L.

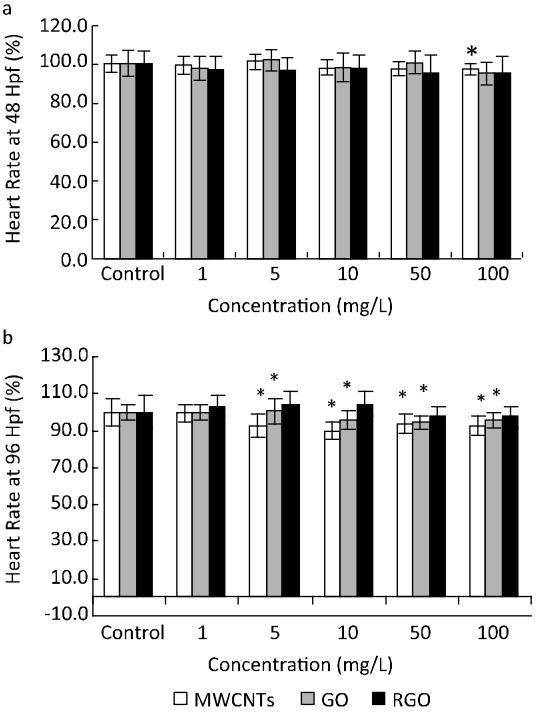

Figure 4. Heart rate of zebrafish embryos at (a) 48 hpf and larvae at (b) 96 hpf exposed to MWCNTs, GO, and RGO at concentrations from 1 to 100 mg/L. *P<0.05 compared with the control.

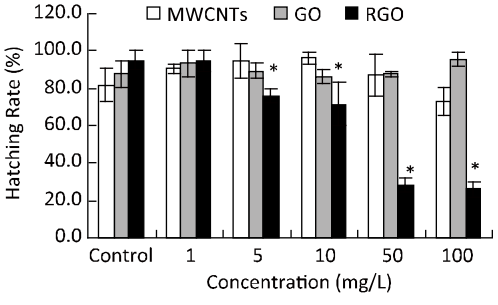

Figure 5. Hatching rate of zebrafish embryos exposed to MWCNTs, GO, RGO at concentrations ranging from 1 to 100 mg/L. *P<0.05 compared with control.

Length of Larvae

To examine the effects of MWCNTs, GO, and RGO on the delay in hatching of the treated zebrafish embryos and the growth of the larvae, the length of larvae was measured at 96 hpf, and the results are shown in Figure 6. GO had no affect on the length of larvae at concentrations ranging from 1 to 100 mg/L. MWCNTs had no significant effect on the larvae length, except at concentration of 100 mg/L. However, RGO had more complex effect on the length of larvae. At a low concentration of 1 mg/L, the length of larvae increased obviously compared with that of the controls (concentration of 0 mg/L). However, at the concentration of 10 mg/L to 100 mg/L, the growth of larvae was inhibited obviously. These results indicated that RGO had greater inhibitory effect on the growth of zebrafish than MWCNTs, and GO had no affect on the growth of larvae.

DISCUSSION

In this study the toxicity of MWCNTs, GO, RGO to the early stages of development of zebrafish were investigated. No abnormality such as tail detachment, somite formation, circulatory system, or pericardial cyst pigmentation was observed in the developmental endpoints in the treated embryos. Furthermore, none of the 3 nanomaterials induced mortality of embryos. Compared with other metallic nanoparticles, although no significant development effects including mortality and abnormities were observed, the sublethal effects on heart rate, hatching rate, or the length of larvae were induced[41]. MWCNTs and GO affected the heart rate, and RGO affected the hatching and the length of larvae in a concentration-dependent manner.

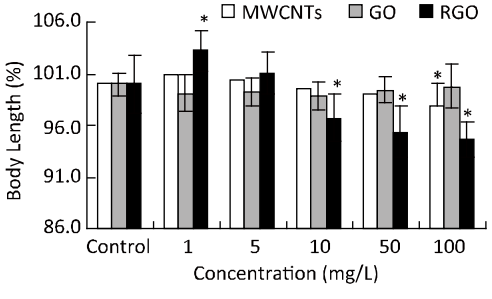

Figure 6. Length of larvae exposed to MWCNTs, GO and RGO at concentrations ranging from 1 to 100 mg/L. $^{*}P<0.05$ compared with control.

To elucidate the differences in the toxicity among the 3 nanomaterials, it is essential to consider the properties of MWCNTs, GO, and RGO. RGO and MWCNTs are hydrophobic, whereas GO is hydrophilic[3,4,42]. The particle size, surface area, and chemical form of the nanomaterials will change when they enter the environment, which is important for their toxicity[43]. Previous studies have reported that addition of nanomaterials like ZnO and CNTs into the standard water for the zebrafish led to formation of aggregates and a change in the original size distribution of these nanomaterials[25,33]. In this study, although GO is hydrophilic, the agglomeration and precipitation of GO were observed, as well as MWCNTs and RGO adhered to the chorion of the zebrafish embryos (Figure 2). MWCNTs and GO had significant effects on heart rate, while RGO had no such efect. The reason for the different effects of MWCNTs, GO, and RGO on the heart rate of zebrafish might be related to their different physical and chemical properties. So further studies are needed to address this issue.

Hatching rate is an important endpoint to determine the effect of nanomaterials. Previous studies[11,25,44] indicated that the hatching rate was significantly reduced by the nanomaterials, including CNTs, ZnO, and CeO2-based catalysts. The delay in hatching of zebrafish embryos might be attributed to the interference with hatching enzyme and hypoxia in zebrafish. The nanomaterials which precipitated and adhered to the chorion of the zebrafish embryos might interfere with the digestive function of hatching enzyme and oxygen exchange[25,35]. Chen[45] investigated the hatching rate under two level of GO, which demonstrated that GO slightly delayed hatching of zebrafish embryos at 50 mg/L. Results from this experiment suggested that RGO significantly reduced the hatching rate, while GO and MWCNTs at concentrations ranging from 1 to 100 mg/L had no effects on the hatching rate. The difference in the effect of these 3 nanomaterials on hatching rate might be due to the differences in the adhesion to the surface of the chorion of zebrafish. Graphene adhered more tightly and more completely to the chorion than MWCNTs and GO (Figure 2). In addition, graphene reduced the body length of larvae. The reasons for growth inhibition might include: a secondary effect of aryl hydrocarbon receptor (AhR)-mediated toxicity caused by reduced blood flow[46] and the consequences of insufficient nutrients required for normal development[47]. Graphene had no affect on

the heart rate of zebrafish and thus the loss of nutrients mightbe responsible for the growth inhibition. Furthermore, the inhibition of growth might be attributed to the difference in the adhesion of the nanomaterials to the chorion of zebrafish embryos.

CONCLUSION

In conclusion, compared with metallic nanoparticles, MWCNTs, GO, and RGO did not show high toxicity to zebrafish embryos, but had some sublethal effects on the heart rate, hatching rate, and the length of larvae. The tests' concentrations ranged from 1 mg/L to 100 mg/L for zebrafish embryos. GO and MWCNTs had significant effects on the heart rate, while RGO affected the embryos hatching and the length of larvae in a dose-dependent manner. In addition, MWCNTs had obvious effect to reduce the length of larvae at high concentrations. In these tests, no abnormality or mortality was found, and MWCNTs, GO, and RGO at the concentration <100 mg/L did not show severe toxicity in the aquatic environment.

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