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Short Communication

DEVELOPMENTAL TOXICITY IN ZEBRAFISH (*DANIO RERIO*) EMBRYOS AFTER EXPOSURE TO MANUFACTURED NANOMATERIALS: BUCKMINSTERFULLERENE AGGREGATES (nC_{60}) AND FULLEROL

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Abstract—The present paper summarizes, to our knowledge, the first study regarding the developmental toxicity of stable buckminsterfullerene aggregates suspended in water (nC_{60}) using zebrafish (*Danio rerio*) as a vertebrate model. Zebrafish embryo survival, hatching rate, heartbeat, and pericardial edema were noted and described within 96 h of exposure. Fullerol (a hydroxylated C_{60} derivative, $C_{60}(OH)_{16–18}$) at 50 mg/L did not exert toxicity to zebrafish embryos. In contrast, nC_{60} at 1.5 mg/L delayed zebrafish embryo and larval development, decreased survival and hatching rates, and caused pericardial edema. Toxicity was mitigated by adding an antioxidant (glutathione), which suggests that a free radical–induced mechanism or another form of oxidative stress played a role in developmental toxicity.

Keywords—Nanolitter Fullerene Zebrafish Embryo development Ecotoxicology

INTRODUCTION

Buckminsterfullerene (C_{60}), in either solid state or organic solution, does not precipitate completely when coming into contact with water. Some C_{60} can form stable, nanoscale, suspended aggregates (nC_{60}), the concentration of which can reach up to 100 mg/L [1]. Thus, their potential ecotoxicological effects in aquatic environments are of great interest. Most studies regarding the biological effects of these water-soluble C_{60} aggregates, however, have focused on biomedical implications, and little is known about the potential ecotoxicity of nC_{60}.

Oberdörster [2] recently published the first report about manufactured nanomaterials (MNMs) imperiling an aquatic organism's health and showed that nC_{60} could exert oxidative stress and cause severe lipid peroxidation in fish brain tissue. Another recent study examining the effect of nC_{60} on freshwater crustaceans noted that after 21 d of exposure, *Daphnia magna* had a delay in molting and a significant decrease in offspring production at 5 mg/L [3]. The mortality of *Daphnia* organisms also was reported to increase with nC_{60} concentration [4]. These studies reflect the importance of considering the potential environmental impacts of such nanolitter.

The early life-stage test using the zebrafish (*Danio rerio*) embryo currently is one of the most widely used tools in environmental science research, especially for investigating the toxicity and teratogenicity of chemicals that could significantly affect environmental health [5]. In the present work, zebrafish was used as a vertebrate model to study the developmental toxicity of nC_{60} and contribute to our understanding of the potential ecotoxicological impacts of MNM releases to aquatic environments.

MATERIALS AND METHODS

MNM preparation

The stock solution of nC_{60} was prepared based on the method described by Scrivens and Tour [6]. Uncoated C_{60} powder (purity, 99.5%; SES, Houston, TX, USA) was first dissolved in benzene, and then 100 μl of this solution were combined with 10 ml of tetrahydrofuran (THF) at room temperature. The resulting mixture was added dropwise to 200 ml of rapidly stirred acetone. Then, 150 ml of Milli-Q® water (Millipore, Billerica, MA, USA) were slowly added to the resulting acetone/THF/benzene/C_{60} solution. Upon complete addition of water, the organic solvents and some water were removed by slow boiling (75°C) to yield the final 50 ml of nC_{60} water suspension. The concentration of nC_{60} produced in the present study was approximately 3 mg/L, and the particles were observed to have an irregular shape, with a typical size of approximately 100 nm (Fig. 1).

Fullerol stock solution was prepared by dissolving 10 mg of $C_{60}(OH)_{16–18}$ (purity, >85%, with <15% $C_{70}(OH)_{16–18}$; a gift of Qingdao University of Science and Technology, Qingdao, China) in 100 ml of Milli-Q water. The concentration of $C_{60}(OH)_{16–18}$ in this solution was 100 mg/L.

Reduced glutathione (GSH; purity, >98.0%; AMRESCO, Solon, OH, USA) solution was prepared by dissolving 3 mg of GSH in 50 ml of reconstituted water. The reconstituted water (used to grow zebrafish) was prepared according to International Organization for Standardization (Geneva, Switzerland) standard 7346-3:1996 with minor alterations [7]. It consisted of 130 mg/L of $NaHCO_3$, 11.5 mg/L of KCl, 246 mg/L of $MgSO \cdot 7H_2O$, and 588 mg/L of $CaCl_2 \cdot 2H_2O$. The stock concentration of GSH was 60 mg/L.

Exposure process

Danio rerio (second generation in our laboratory, originally obtained from an aquarium in Tianjin, China) were tested using the embryonic toxicity test developed by Schulte and Nagel [5]. The test was started as soon as the intact, fertilized *D. rerio* eggs were selected (within 1.5 h postfertilization [hpf]). Twenty-four eggs (blastula stage) were transferred into the test

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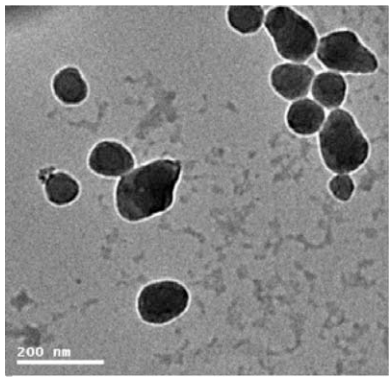

Fig. 1. Transmission-electron microscopic image of nC_{60}. The average cluster size was approximately 100 nm.

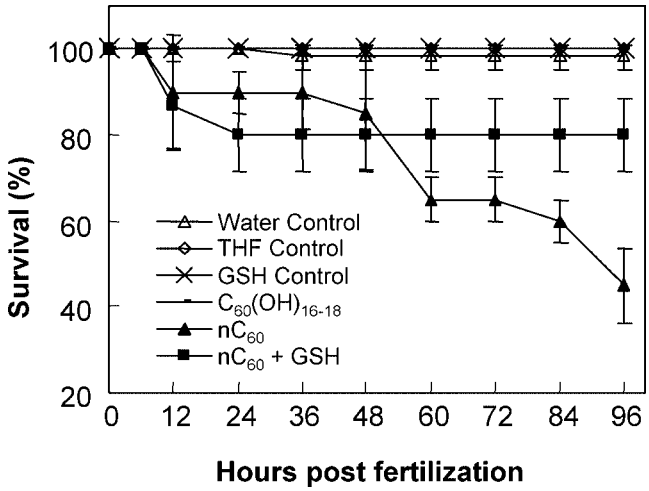

Fig. 2. Effects of different treatments on survival of zebrafish (*Danio rerio*) embryos during a 96-h time course. Treatments included 1.5 mg/L of nC_{60}, 50 mg/L of $C_{60}(OH)_{16–18}$, and the solvent controls. The antioxidant experiment had 30 mg/L of reduced glutathione (GSH) and 1.5 mg/L of nC_{60}. Dead embryos were removed daily. Data were averaged from triplicate experiments ($n = 60$ embryos for each treatment, except for the water control with 12 embryos) and are shown as the mean ± standard deviation.

wells of a 24-well multiplate so that each well contained one embryo. Twenty wells were prepared with 1 ml of MNM (treatment) solution and 1 ml of reconstituted water each. The remaining four (water-control) wells were prepared similarly, but with Milli-Q water replacing the treatment solution (1 ml of reconstituted water + 1 ml of Milli-Q water). The final concentrations of nC_{60} and $C_{60}(OH)_{16–18}$ were 1.5 and 50 mg/L, respectively. Additional controls were prepared to discern the potential toxicity of residual solvents. These controls were amended with THF at concentrations of up to 144 mg/L, which exceeds by several orders of magnitude the maximum residual solvent concentration based on total organic carbon measurements. The wells were covered with transparent plastic film and placed in an incubator at 26 ± 1°C with a 14:10-h light:dark photoperiod.

Antioxidant test

Fullerene toxicity to fish has been associated with oxidative stress [2]. Thus, a 24-well multiplate was used to investigate if GSH, a known antioxidant [8], could mitigate toxicity. Twenty wells were prepared with 1 ml (each) of GSH and nC_{60} solution (1.5 mg/L). The other four (control) wells were prepared similarly, but with Milli-Q water replacing the nC_{60} solution. The final concentration of GSH was 30 mg/L (0.1 mM). This concentration is comparable to that used by Song et al. [9], who found that 0.2 mM GSH protected hippocampal neuron cells from the toxic effects of H_2O_2-derived hydroxyl radicals, whereas a higher dose of 20 mM had a diminished beneficial effect.

Embryolarval toxicity test

The development status of zebrafish embryos and larvae were observed with an inverse microscope (×8–50; IMT 2; Olympus, Tokyo, Japan) and documented photographically at specified times (6, 12, 24, 36, 48, 60, 72, 84, and 96 hpf). End points used for assessing developmental toxicity included embryo survival, hatching rate, heartbeat (visually determined as the average for five embryos, counted for 15 s under a temperature-controlled stage), and pericardial edema and were described for embryos and larvae from both control and treated groups.

Statistical analysis

All experiments were repeated three times independently. Statistical analyses were carried out using analysis of variance (ANOVA), and data were illustrated as the mean with standard deviation (SD). A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Survival

The survival of zebrafish embryos exposed to different treatments was determined at specified time points. As shown in Figure 2, 50 mg/L of $C_{60}(OH)_{16–18}$ and the solvent controls exerted no detectable toxicity to zebrafish embryos or larvae, whereas 1.5 mg/L of nC_{60} was toxic. At 96 hpf, the survival of embryos was 45%, which suggests that the zebrafish developmental toxicity of nC_{60} got stronger with exposure duration.

Additional treatments amended with nC_{60} and GSH were tested. Glutathione mitigated the developmental toxicity exerted by nC_{60}, increasing the survival of the embryos and larvae to 80% after 24 h of exposure (Fig. 2). Spectroscopic analysis of nC_{60} concentrations (at 336 nm) with and without GSH indicated that addition of GSH did not induce nC_{60} precipitation or reduce its bioavailability [10]. Considering that nC_{60} has been reported to exert oxidative stress in fish tissue [2] and human cell lines [1], we postulate that the beneficial effect of GSH resulted from to its antioxidant properties that mitigated oxidative stress.

Hatching rate

Compared to the no-treatment controls, $C_{60}(OH)_{16–18}$ and the solvent controls did not significantly ($p > 0.05$) affect the hatching rate during the 96-h exposure time (Fig. 3). In contrast, nC_{60} displayed notable embryonic developmental delay and toxicity. The hatching rates were 44% in the water control and approximately 25% in both solvent controls at 60 hpf, whereas that of nC_{60} was 0%. At 84 hpf, the hatching rate of the set exposed to nC_{60} peaked and leveled off at 15%. In contrast, the treatment with nC_{60} plus GSH exhibited a 35% hatching rate at 60 hpf, which was similar to that of control groups, and it increased to 70% at 84 hpf. This final value is

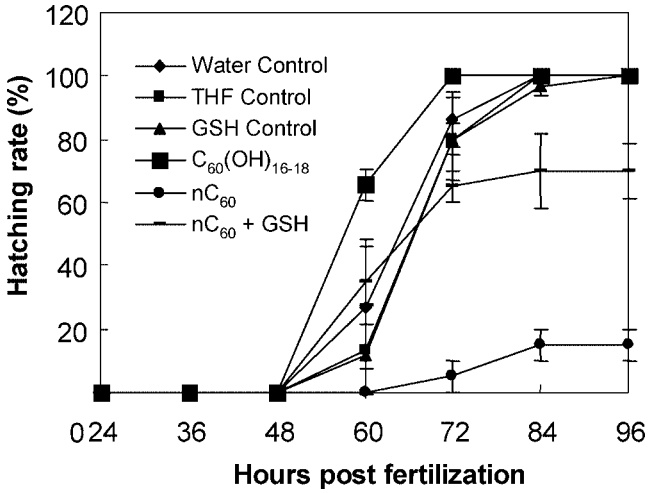

Fig. 3. Hatching rate (%) of zebrafish (*Danio rerio*) embryos exposed to different treatments (30 mg/L of reduced glutathione (GSH), 50 mg/L of $C_{60}(OH)_{16–18}$, 1.5 mg/L of nC_{60}, and 1.5 mg/L of nC_{60} + 30 mg/L of GSH) during 96 h. Error bars represent one standard deviation from the mean of three replicates (n = 60 embryos).

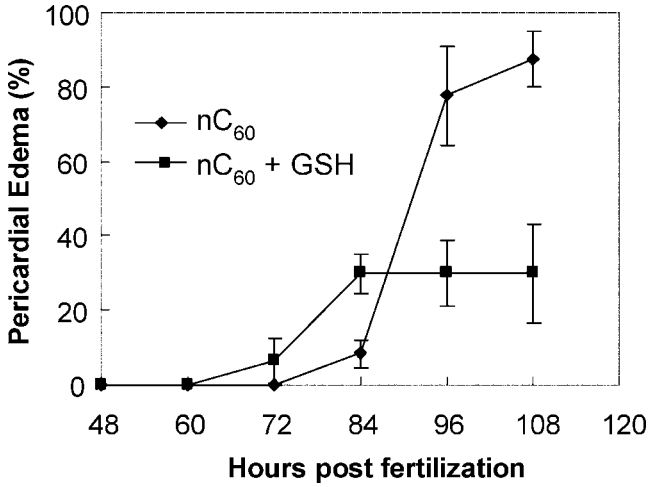

Fig. 4. Mitigation of nC_{60}-induced pericardial edema (1.5 mg/L of nC_{60}) by the presence of reduced glutathione (GSH; 30 mg/L). Error bars represent one standard deviation from the mean of three replicates (n = 60 embryos).

lower than that of the water control but significantly higher than that of nC_{60} ($p < 0.05$), and it corroborates the idea that GSH could effectively mitigate the developmental toxicity of nC_{60}.

Heartbeat and pericardial edema

Altered heartbeat and pericardial edema were not observed in zebrafish embryos or larvae exposed to $C_{60}(OH)_{16–18}$ or solvent controls. For the 1.5 mg/L of nC_{60} treatment, the following phenomenon was observed: At 12 to 48 hpf, fish survival decreased from 90 to 60%, whereas the heartbeat in the surviving embryos (70–80 beats/min) was slower than that in controls (140–160 beats/min). At 84 hpf, pericardial edema was observed in unhatched embryos and larvae, affecting 8.3% of the surviving fish; this percentage increased sharply to 77.8% at 96 hpf (Fig. 4). Incardona et al. [11] reported that the pericardial edema of zebrafish embryos probably was caused by the insufficient production of cardiac troponin T.

The antioxidant test results showed no slowed heartbeat before 48 hpf. Although pericardial edema began to emerge at 72 hpf in some of the fish, the proportion of surviving embryos and larvae with pericardial edema leveled off at 30% (Fig. 4). These patterns corroborate survival and hatching rate results showing that GSH mitigates the developmental toxicity of nC_{60}.

DISCUSSION

At the single concentrations tested, nC_{60} (1.5 mg/L) exerted distinct developmental toxicity (exhibited progressively by decreased heart rate, pericardial edema, and ultimately, death), whereas $C_{60}(OH)_{16–18}$ (50 mg/L) did not. A nC_{60} suspension prepared by Sayes et al. [1], which was prepared similarly, but with THF as the transitional solvent, also displayed strong biotoxicity to human cells (i.e., it was toxic to human skin fibroblast at 0.02 mg/L). This material also was toxic to juvenile largemouth bass at 0.5 mg/L, causing lipid peroxidation in brain tissue [2]. Andrievsky et al. [12] postulated that the toxicity of nC_{60} observed in the above studies was primarily the result of impurities, such as residual THF or its degradation products (accounting for 10% [weight:weight]), rather than of the nC_{60} aggregate itself. Recent studies, however, have shown that nC_{60} prepared by long-term stirring of C_{60} powder in water exhibits bactericidal properties similar to those of solutions prepared with intermediate solvents or solubilizers [13] and that, similar to the present results (Fig. 2), residual solvent controls did not exert microbial toxicity [14]. Lovern and Klaper [4] also recently concluded that residual THF was not a influential factor in the toxicity of nC_{60} to *Daphnia* sp.

Structurally, $C_{60}(OH)_{16–18}$ is a derivative of C_{60} with 16 to 18 hydroxide radicals groups connected by covalent bonds. This fullerol had no detectable developmental toxicity to zebrafish embryos, similar to the results of other cytotoxicity experiments [1,13,14]. Apparently, toxicity decreases as the number of chemical groups attached to C_{60} (and their attachment symmetry) increases [1]. The fact that MNMs differing in structure exert different levels of developmental toxicity suggests that mechanistic research on structure–reactivity–toxicity relationships might be a fruitful avenue to identify manufacturing and derivatization approaches that decrease the potential ecotoxicity of nanolitter. It also is recommended that exposure experiments be conducted using nC_{60} prepared by different methods over a broader range of nC_{60} concentrations to characterize dose–response relationships. Such studies will be particularly important in the assessment of nanomaterial risks, because toxicants that affect embryonic development exert their effects at lower concentrations than those required to affect adults or to cause general cytotoxicity. Thus, the determination of developmental toxicity thresholds will help to develop water-quality standards to protect aquatic life.

The developmental toxicity of nC_{60} was effectively attenuated by adding GSH, an antioxidant. Glutathione has sulfide functional groups that can capture unpaired electrons and is thus capable of removing harmful free radicals [14,15]. The beneficial presence of GSH supports the notion that there might be a free radical–induced toxicity mechanism in the developmental toxicity of nC_{60}. Nonetheless, GSH did not completely prevent embryo damage (Figs. 3 and 4). Further research is needed to determine whether the inability of this antioxidant to provide full protection resulted from an insufficient quantity being added or from the fact that other toxicity mechanisms besides oxidative stress also may be important.

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