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### EFFECT OF CARBON NANOTUBES ON DEVELOPING ZEBRAFISH (DANIO RERIO) EMBRYOS

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**Abstract**—The impact of carbon nanotubes (CNTs) on the aquatic environment was investigated by examining the properties of raw CNTs under several environmental conditions and using developing zebrafish (*Danio rerio*) embryos. The agglomerate size for single-walled CNTs (SWCNTs) was significantly larger at pH 11 or greater and was stable at temperatures from 4 to 40°C and salinities from 0 to 30 ppt. Exposure to SWCNTs induced a significant hatching delay in zebrafish embryos between 52 to 72 h postfertilization (hpf) at concentrations of greater than 120 mg/L, but 99% of the exposed embryos hatched by 75 hpf. Double-walled CNTs also induced a hatching delay at concentrations of greater than 240 mg/L, but carbon black did not affect hatching at the concentrations tested. Molecular and cellular analysis showed that the embryonic development of the exposed embryos up to 96 hpf was not affected at SWCNT concentrations of up to 360 mg/L. Scanning-electron microscopic inspection showed that the size of the pores on the embryo chorion was nanoscaled and that the size of SWCNT agglomerates. The hatching delay observed in this study likely was induced by the Co and Ni catalysts used in the production of SWCNTs that remained at trace concentrations after purification. This study suggests that materials associated with raw SWCNTs (perhaps metal contaminants) have the potential to affect aquatic life when released into the aquatic environment.

Keywords—Carbon nanotubes

Zebrafish embryos

Hatching delay Chorion

Nanomaterials

#### **INTRODUCTION**

Nanomaterials are part of an industrial revolution to develop lightweight, strong materials for a variety of purposes. Carbon nanotubes (CNTs), which are nano-sized, hollow, graphite cylinders, were developed in 1991 by Iijima [1]. The unique physical and chemical properties of CNTs have raised great expectations regarding their use for numerous applications in medicine, chemistry, electronics, materials, and the environment [2], and many novel discoveries and applications of CNTs have been reported in recent years [2–7]. As the production and application of CNTs increase, the introduction of CNTs into the environment also will occur more frequently. Therefore, it is important to determine the health and environmental impact as well as the potential hazardous effects of CNTs and associated nanomaterials in the environment before these compounds are in widespread use [8–11].

There are two main types of CNTs, single-walled CNTs (SWCNTs) and multiwalled CNTS (MWCNTs). These types are determined according to differences in structure and diameter. The synthesis methods for SWCNTs and MWCNTs mainly are carbon-arc discharge, carbon laser ablation, or chemical vapor deposition (CVD). Arc discharge and CVD synthesis are the most common techniques that are used to produce SWCNTs, because the laser ablation method is expensive. The CVD method commonly is used to generate double-walled CNTs (DWCNTs) but also MWCNTs in general.

After certain purification and functionalization procedures, raw CNTs can be modified to functionalized CNTs, which have a larger spectrum of biomedical applications [7,12] and which differ from raw CNTs in compatibility and toxicity as a result of differences in their surface properties and purities. Uncoated, raw CNTs are strongly hydrophobic, whereas oxidation-purified CNTs have functional groups on their surfaces that make them more hydrophilic [12]. This change can affect their toxicity. A previous study using 3T3 fibroblasts [13] suggested that the removal of production contaminants and the reduction of SWCNT hydrophobicity with the addition of carboxylic acid groups played a positive role in cell-SWCNT interactions. Although cell viability decreased on exposure to raw SWCNTs, a comparatively significant increase in cell viability was found when 3T3 fibroblasts were incubated with purified SWCNTs or glucosamine-functionalized SWCNTs. Another experiment also demonstrated that as the degree of sidewall functionalization increases, SWCNTs become less cytotoxic, indicating that the cytotoxic response of cells in culture is partially dependent on the degree of SWCNT functionalization and purity [14]. Oxidized MWCNTs, however, were more toxic than raw MWCNTs and induced mass loss of cell viability through programmed cell death in human T cells [15].

Raw MWCNTs also were demonstrated both to localize within and to initiate an irritation response in the form of release of the proinflammatory cytokine interleukin-8 in human keratinocytes [16]. Ding et al. [17] reported that exposure to raw MWCNTs activated genes involved in cellular transport, metabolism, cell-cycle regulation, and stress response in human fibroblasts, indicating a strong immune and inflammatory response [17].

Carbon nanotubes with different geometric structures also exhibit quite different cytotoxicity and bioactivity in vitro. Raw SWCNTs were found to be more toxic than raw MWCNTs [18]. In human keratinocyte cells, exposure to SWCNTs resulted in increased oxidative stress and inhibition of cell proliferation, and it was suggested that SWCNTs might activate nuclear factor- $\kappa$ B in a dose-dependent manner because of the activation of stress-related kinases [19]. Experiments using human serum samples indicated that inflammation and granuloma formation might be caused by the damaging effects of excessive complement activation, because CNTs activated human complement via both classical and alternative pathways [20].

Beyond in vitro cytotoxicity experiments, most of the currently published research has focused on the respiratory and inhalation toxicity of nanomaterials [9]. Because unprocessed CNTs are very light, they could potentially become airborne and reach the lungs [21]. Both MWCNTs and ground CNTs were found to induce inflammatory and fibrotic responses and to stimulate the production of tumor necrosis factor  $\alpha$  in the lungs of treated rats [22]. In experiments with mice, SWCNTs induced dose-dependent epithelioid granulomas and interstitial inflammation in treated mice after a 7-d exposure, and these lesions persisted and were more pronounced after 90 d of exposure [21]. Similarly, pharyngeal aspiration of SWCNTs elicited unusual pulmonary effects in another mouse strain that combined robust and acute inflammation with early onset yet progressive fibrosis and granulomas [23].

Most CNT products ultimately will enter the aquatic environment, which could profoundly influence both human and environmental health [9,24]. As production increases and costs decrease, CNTs and, especially, SWCNTs will become more easily available in large amounts. For example, in China, the production capacity of CNTs has greatly increased. One leading company can produce 10 tons of CNTs in one year (http://www.timesnano.com/info/introduce.html), and the number of these companies is increasing because of the huge potential for commercial interest and success. At the same time, the price of CNTs has dropped dramatically. Once CNTs are produced cheaply and in large amounts, they will be used more widely around the world and, as a consequence, could profoundly influence the aquatic environment. For example, some of the CNTs that enter the aquatic environment will come to be part of the sediments in aquatic systems and influence water quality [25]. Little, however, is known about the interaction of CNTs with the aquatic environment. A recent study in juvenile largemouth bass indicated that C<sub>60</sub> fullerene prepared in tetrahydrofuran caused oxidative damage and depletion of glutathione [8]. The C<sub>60</sub> fullerene is a spherical molecule, whereas CNTs are elongated molecules with a diameter of several nanometers and a length of up to several micrometers. Both C<sub>60</sub> fullerene and CNTs are carbon nanomaterials. To validate the biocompatibility and safety of introducing CNTs into the aquatic environment, we have explored the behavior of raw SWCNTs under various environmental conditions and their effects on zebrafish embryos.

Raw SWCNTs tend to aggregate in solutions, because they are subject to substantial van der Waals attractions. Because the presence of salts and changes in pH in the natural aquatic environment might alter their physical behavior, we investigated the impact of environmental factors on the particle size distribution of SWCNTs to predict possible changes in the toxicity of these compounds under different aquatic conditions. We also tested DWCNTs and carbon black to make comparisons with other carbon nanomaterials.

Our objective was to study the effects of SWCNT exposure on embryonic development of zebrafish at both the gross mor-

phological and the molecular level. Hatching success and timeto-hatch were observed, and body length and head-trunk angle were examined at several important time points as the development index. In addition, live embryos were stained with acridine orange to measure cell death resulting from SWCNT exposure. We examined the interaction between the chorion and raw SWCNTs using scanning-electron microscopy, because the chorion is the first protective barrier for the embryos. The influence of raw SWCNT exposure on several zebrafish developmental regulatory factors-MyoD, Ngn 1, Zash 1a, and Zash 1b-were investigated at the molecular level with wholemount in situ hybridization and immunohistochemistry. Because CNTs were found to disperse in the serum of several model organisms through nonspecific binding interactions with biological molecules [4,26], potential CNT effects on the circulatory system of zebrafish embryos were assessed using endogenous alkaline phosphatase activity staining of the blood vessels at 72 hpf.

#### MATERIALS AND METHODS

#### Raw CNTs

Raw SWCNTs (Sigma-Aldrich, St. Louis, MO, USA) were produced by the arc discharge method in the presence of Co and Ni as metal catalysts and then purified by acid leach and thermal oxidation. The purity, as determined by energy dispersive x-ray spectroscopy, was approximately 90 atom percent; the average diameter was 11 nm and the length 0.5 to 100  $\mu$ m. Double-walled CNTs were synthesized by the catalytic CVD method using a catalyst of Mg<sub>1-x</sub>Co<sub>x</sub>O solid solution containing small additions of Mo oxide (Mo<sub>x</sub>O<sub>y</sub>), and the purity after removing the catalysts was approximately 98 atom percent [27]. Carbon black (Cabot Corporation, Billerica, MA, USA) was produced by the channel method, and the average aggregate size was in the range of 0.01 to 1.0  $\mu$ m.

# Particle size distribution of SWCNT agglomerates in the aquatic environment

Single-walled CNTs with a concentration of 50 mg/L were dispersed by stirring with a magnetic stir bar for 30 min in freshwater and seawater (salinity, 30 ppt) using combinations of pH values (3, 5, 7, 9, and 11) and temperatures (4, 16, 25, and 40°C). Light microscopic images were then collected from five randomly selected, 6.85-mm<sup>2</sup> regions of each combination of conditions using a cooled color digital camera (TK-C1381; Victor Company of Japan, Yokohama, Japan) combined with an inverted microscope (Eclipse TE200; Nikon, Tokyo, Japan). The particle size and number of the SWCNT agglomerates were calculated and counted digitally and automatically using the MetaMorph software (Ver 5.0r2; Universal Imaging, Sunnyvale, CA, USA). Particle size was log-transformed and categorized into six levels. The distribution of the particle sizes was then calculated and determined using Microsoft Excel® (Microsoft, Redmond, CA, USA).

#### Zebrafish maintenance and experimental setup

Mature zebrafish were purchased from a local commercial source (Chong Hing Aquarium, Hong Kong, China). The zebrafish colony was maintained as described previously [28]. Embryos were obtained by photo-induced spawning over green plants and then cultured at 28.5°C in filtered tap water. The developmental age of the embryos was measured according to hpf and staged according to the method described by Kimmel et al. [29]. At 4 hpf, embryos were examined under a dissecting microscope, and only embryos that developed normally and reached the blastula stage were selected for subsequent experiments.

The dispersion of SWCNTs was carried out by stirring with a magnetic stir bar for 30 min in the filtered tap water. The selected 4-hpf zebrafish embryos were exposed to six concentrations of SWCNTs (20, 40, 60, 120, 240, and 360 mg/L) dispersed in filtered tap water and a filtered tap water control (pH 7, 28.5°C). Fifteen replicates were set up for each concentration; each replicate consisted of 20 embryos exposed to 6 ml of testing medium in a Petri dish (diameter, 55 mm; n = 300 for each level of treatment). Exposure to SWCNTs lasted from 4 to 96 hpf. The embryos in each replicate were assessed using a dissecting stereomicroscope at ×60 magnification at 24, 48, 52, 56, 72, 75, and 96 hpf, and the number of surviving and hatching embryos in each replicate was recorded at all time points.

Both because a hatching delay was observed in the SWCNT exposure and to make comparisons with other carbon nanomaterials, a second group of 4-hpf zebrafish embryos was exposed to 120 and 240 mg/L of each nanomaterial (SWCNTs, DWCNTs, and carbon black). Ten replicates were set up for each concentration, consisting of 20 embryos exposed to 6 ml of test medium in a Petri dish (diameter, 55 mm; n = 200 for each level of treatment). The exposure period in this experiment also was from 4 to 96 hpf.

#### Measurement of the head-trunk angle and body length

The head-trunk angle of the embryos exposed to 240 mg/L of SWCNTs was measured in five embryos selected randomly from each of the five replicates at 24 and 48 hpf, and the body length of the exposed embryos was examined at 48 hpf in another group of five embryos selected randomly from each of the five replicates. The dechorionated embryos were anesthetized with 0.016 M tricaine (Sigma-Aldrich), mounted in lateral view in 0.5% agarose (Invitrogen, Carlsbad, CA, USA) in culture medium on clean slides, and then photographed using a camera mounted on a stereomicroscope (SZX-12; Olympus, Tokyo, Japan). The pictures were then analyzed with Metamorph software.

### Cell-death analysis using acridine orange staining of live embryos

Cell death in embryos exposed to 240 mg/L of SWCNTs was analyzed using acridine orange staining. An amount of 0.003% (w/v) 1-phenyl-2-thiourea (PTU; Sigma-Aldrich) was applied to each treatment at the beginning to suppress the expression of pigment [30] in the embryos. The dechorionated zebrafish embryos were collected at 42 hpf and then incubated in 0.005% acridine orange (Molecular Probes, Eugene, OR, USA) in embryo medium for 20 min and washed three times with filtered tap water for 5 min each. Before examination, embryos were anesthetized with 0.016 M tricaine (Sigma-Aldrich). Photographs of each embryo were taken with a confocal microscope (Laser Scanning Microscope 410; Carl Zeiss, Jena, Germany).

#### Blood vessel staining

Blood vessel formation was analyzed using blood vessel staining according to the method described by Serbedzija et al. [31]. At 72 hpf, the embryos exposed to 240 mg/L of SWCNTs were collected and then fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) con-

taining 1% Triton X-100 (Sigma-Aldrich) before being stained for endogenous alkaline phosphatase activity. For staining, embryos were equilibrated in TMNT buffer (0.1 M Tris-HCl [pH 9.5], 50 mM MgCl<sub>2</sub>, 0.1 M NaCl, and 0.1% Tween 20) at room temperature three times for 10 min. Once the embryos equilibrated in TMNT, 4.5  $\mu$ l/ml of 75 g/L of nitro blue tetrazolium and 3.5  $\mu$ l/ml of 50 g/L of 5-bromo-4-chloroindolyl phosphate (DAKO, Glostrup, Denmark) were added. After staining for 10 min, all the blood vessels in the fish embryo were labeled. The staining reaction was stopped by washing in PBS containing 0.1% Tween 20 (PBT). Embryos were then immersed in a solution of 5% formamide and 10% H<sub>2</sub>O<sub>2</sub> in PBS for 20 min, which removed endogenous melanin in the pigment cells and allowed full visualization of the stained vessels.

#### Whole-mount in situ hybridization

Several molecular markers were analyzed at 24 hpf in embryos exposed to 240 mg/L of SWCNTs using whole-mount in situ hybridization according to the method described by Westerfield [30]. Antisense RNA for MyoD, Ngn 1, Zash 1a, and Zash 1b was synthesized by linearizing the plasmids and transcribing with T7 or SP6 polymerase and digoxigenin-11-2'-uridine-5'-triphosphate (Boehringer, Indianapolis, IN, USA). Embryos were fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS at 4°C overnight, dehydrated in a methanol (MeOH) series, and stored in absolute MeOH at -20°C to increase permeability. Embryos were then digested with Proteinase K (Sigma-Aldrich) and refixed before incubation with the antisense probes at 65°C overnight. Probes were removed with high-stringency washes at 65°C. Embryos were then incubated with preabsorbed goat serum (Sigma-Aldrich) for 4 h at room temperature. After washing, 5-bromo-4-chloroindolyl phosphate was added as the substrate, and nitro blue tetrazolium (DAKO) was added as the coupler. The SWNT-treated and control embryos were examined using an inverted microscope (Eclipse TE200). Images of SWCNT-treated and control embryos were captured with a cooled color digital camera (TK-C1381; Victor Company of Japan), and then data were collected and stored using a digital camera attached to a computer and Metamorph software.

#### Whole-mount immunostaining

Embryos exposed to 240 mg/L of SWCNTs were examined for neurogenesis with whole-mount immunostaining at 52 hpf according to the method described by Westerfield [30]. Embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS containing 1% Triton X-100. Fixed embryos were washed four times in PBT and dehydrated by immersion in 25, 50, 75, and 100% methanol in PBT to permeabilize the embryos. Embryos were treated in 10% H2O2 (BDH Laboratory Supplies, Poole, UK) in MeOH at 4°C for 1 h to permeabilize the tissues before they were rehydrated stepwise to 100% PBT. Embryos were then incubated with 5% goat serum in PBT for 1 h at room temperature to block nonspecific binding sites and, subsequently, were incubated in mouse anti-Zn5 (1:100; Developmental Biology Hybridoma Bank, Iowa City, IA, USA) with 5% goat serum in PBT at 4°C overnight. After thorough washing, embryos were incubated with biotinylated goat antimouse secondary antibody at 4°C overnight. Horseradish peroxidase-streptavidin was added before incubation of embryos with chromogenic substrate (diaminobenzidine and  $H_2O_2$ ).



Fig. 1. Particle size distribution (PSD) of 50 mg/L of single-walled carbon nanotubes agglomerates in freshwater (A) and seawater (B). The seawater salinity was 30 ppt, and the temperature was 25°C. The particle size distribution of single-walled carbon nanotube agglomerates shifted toward more in the larger size category and fewer in the smaller size category at pH 11 or greater and was stable at other pH values. An asterisk indicates a significant difference (analysis of variance, p < 0.05) compared with all other conditions.

#### Statistical analysis

All data are presented as the mean  $\pm$  standard error from three to six experiments. Significant differences between mean values were determined using two-way analysis of variance (ANOVA) and Tukey's post hoc test. The level of statistical significance in all cases was p < 0.05. Percentage data were log-transformed before analysis. The ANOVA was performed using SigmaStat 3.0 software (SPSS, Chicago, IL, USA).

#### RESULTS

#### Particle size distribution of SWCNT agglomerates

Figure 1 shows the influence of pH on the particle size distribution of SWCNT agglomerates in both freshwater and seawater. The particle size distribution of SWCNT agglomerates was quite stable within an environmental pH range (pH 5–9), and no significant change was found in the particle size distribution with changes in temperature and salinity. In the pH range tested, most of the SWCNT agglomerates were mi-

croscaled, with approximately 8% of the SWCNT agglomerates in the range of 0 to 10  $\mu$ m<sup>2</sup>, 50% in the range of 10 to 10<sup>2</sup>  $\mu$ m<sup>2</sup>, 35% in the range of 10<sup>2</sup> to 10<sup>3</sup>  $\mu$ m<sup>2</sup>, and 7% in the range of 10<sup>3</sup> to 10<sup>4</sup>  $\mu$ m<sup>2</sup> (Fig. 1). All subsequent exposure experiments were carried out in the environmental pH range (pH 5–9) in the present study, so the SWCNT agglomerates were similar in particle size distribution. The particle size distribution at pH 11 was significantly different from that at the other pH levels tested both in freshwater and seawater (AN-OVA, p = 0.042 for 0–10  $\mu$ m<sup>2</sup> SWCNT agglomerates, p <0.001 for 10–10<sup>4</sup>  $\mu$ m<sup>2</sup> agglomerates). The distribution shifted toward larger sizes, with more of the agglomerates in the largest size category and fewer in the smaller size category (Fig. 1).

#### Zebrafish chorion and small SWCNT agglomerates

The chorion protects the embryos and is the first barrier to come into contact with SWCNT agglomerates on exposure. Using scanning-electron microscopy, we observed that the outer layer of the chorion of the zebrafish embryos was covered with protuberances of random sizes and that many small, evenly sized pores were distributed on the inner layer of the chorion (Fig. 2A). The particle size of SWCNT agglomerates observed in embryo culture medium was consistent with the particle size distribution of SWCNT agglomerates observed under aquatic environmental conditions in our previous experiment. We focused on the smaller-sized SWCNT agglomerates under scanning-electron microscopy to compare the sizes of the chorion pores and the agglomerates. The size of the protuberances ranged from several hundred nanometers to several micrometers (Fig. 2B), but the size of all the pores was in the nanoscale (Fig. 2C). The pores on the chorion are necessary for oxygen and nutrient transportation from the outer aquatic environment to the embryo and for the elimination of wastes. Even the size of smaller SWCNT agglomerates observed in embryo culture medium (pH 7, 20°C), however, ranged from several to several hundred micrometers (Fig. 2D and E). When the embryos exposed to SWCNTs were dechorionated manually in embryo culture medium containing SWCNT agglomerates, the agglomerates could adhere stably to the inner layer of the detached chorion, and the size of the SWCNT agglomerate obviously was much larger than the size of the pores on the chorion (Fig. 2F). Under normal exposure conditions, however, SWCNT agglomerates could adhere only to the outer layer of the chorion of the zebrafish embryos and were too large to enter the chorion (Supplementary Fig. 1; SETAC Supplemental Data Archive, Item ETC-26-04-005; http:// www.setacjournals.org).

#### Hatching delay of zebrafish embryos

The hatching period is one of the seven broad periods of zebrafish embryogenesis [29]. When incubated at the normalized, 28.5°C freshwater conditions, zebrafish embryos hatched out of the chorion during the third day of the exposure between 48 and 72 hpf. No embryo mortality was observed in any treatment during the time period tested. Following exposure to 120, 240, and 360 mg/L of SWCNTs from the blastula period, zebrafish larvae demonstrated significantly delayed hatching compared with the control group (Fig. 3A) (ANOVA: p = 0.010, p = 0.001, and p < 0.001 at 52 hpf, p = 0.003, p = 0.002, and p < 0.001 at 56 hpf, and p = 0.250, p = 0.070, and p = 0.020 at 72 hpf for 120, 240, and 360



Fig. 2. The chorion of zebrafish (*Danio rerio*) embryos and single-walled carbon nanotube agglomerates (SWCNTs) at 24 h postfertilization (hpf) viewed under an environmental scanning-electron microscope. The chorion of zebrafish embryos at 24 hpf was covered with protuberances (dark arrow in **A** and white arrow in **B**) on the outer layer and pores on the inner layer (**C**). The size of the protuberances (white arrow in **B**) was from 0.16 to 6.22  $\mu$ m<sup>2</sup>, and the size of the pores (white arrow in **B** and **C**) was approximately 0.17  $\mu$ m<sup>2</sup>. The SWCNTs form agglomerates in water (white arrow in **D** and **E**). Agglomerate sizes in **D** and **E** were approximately 17.43 and 4.05  $\mu$ m<sup>2</sup>, respectively. The SWCNT agglomerates adhered to both the outer and inner chorion, but it is unlikely that SWCNT agglomerates with a size of 46.81  $\mu$ m<sup>2</sup> (white arrow in **F**) or larger than 0.17  $\mu$ m<sup>2</sup> will enter the chorion. GSE = gaseous secondary electron; WD = working distance from the surface of the sample. Bar = 5  $\mu$ m (**A**), 20  $\mu$ m (**B**), 10  $\mu$ m (**C** and **F**), 2  $\mu$ m (**D**), and 1  $\mu$ m (**E**).

mg/L, respectively, of SWCNTs). The hatching delay occurred primarily between 52 and 56 hpf (Fig. 3A and B), sometimes extending to 72 hpf (Fig. 3B and C). The lowest-observedeffect concentration for the hatching delay effect was 120 mg/L for SWCNTs (Fig. 3B) (ANOVA: p = 0.025 at 52 hpf and p = 0.030 at 56 hpf), and 240 mg/L for DWCNTs (Fig. 3C) (ANOVA: p < 0.001 at 56 and 72 hpf for both SWCNTs and DWCNTs). The embryos exposed to higher concentrations of SWCNTs showed a more severe hatching delay at 52 hpf, illustrating the dose-dependent hatching delay at this time point. No obvious dose-response effect of hatching delay, however, was observed during the entire hatching period (Fig. 3A). Interestingly, the hatching delay effect did not influence the hatching success rate and survival of the exposed embryos, and all the exposed embryos hatched out by 72 hpf. Carbon black did not show any influence on hatching at either of the tested concentrations (Fig. 3B and C).

#### Head-trunk angle and body length

To investigate the influence of the hatching delay on the exposed zebrafish embryos, two other developmental parameters were examined. The head–trunk angle, or the angle between a line drawn through the middle of the ear and eye and a second line parallel to the notochord in the midtrunk region (myotomes 5–10), and the body length of zebrafish embryos were used as an index of development at 28.5°C [30]. Because the head–trunk angle increases between 20 and 70 hpf as a consequence of the straightening of the embryo, this end point was used as a function of hours of development. The mean head–trunk angle of the embryos exposed to 240 mg/L of SWCNTs at 24 and 48 hpf was not significantly different from that of the control and carbon black–exposure groups (Fig. 4A), indicating that the development of the zebrafish embryos

exposed to 240 mg/L of SWCNTs at 24 and 48 hpf was not delayed, even though hatching was. Exposure to 240 mg/L of SWCNTs also did not influence the body length of the exposed embryos at 48 hpf (Fig. 4B). Both the head-trunk angle and body length experiment demonstrated that the development of the embryos exposed to 240 mg/L of SWCNTs was not significantly affected.

#### Cell death analysis

Cell death in the exposed embryos was investigated with acridine orange fluorescent staining, and the results indicated that exposure to SWCNTs did not induce cell death at 42 hpf compared with the control (Supplementary Fig. 2; SETAC Supplemental Data Archive, Item ETC-26-04-005; http://www.setacjournals.org). Embryos exposed to SWCNTs, however, developed pigment normally, even with the addition of PTU, a chemical that is well-known (and widely used) to inhibit pigment formation in zebrafish embryos through the inhibition of tyrosinase activity (Fig. 5).

#### Blood vessels

It is known that endogenous alkaline phosphatase activity in zebrafish embryos is not detectable until 48 hpf and is strongly detectable at 72 hpf. The results showed that the formation of the main blood vessels of the embryos exposed to 240 mg/L SWCNTs at 72 hpf was not different from control embryos (Fig. 6).

#### Developmental regulatory factors

The effects of SWCNT exposure in embryos also were investigated at the molecular and cellular levels. Several major organs were investigated further using whole-mount in situ hybridization and immunostaining methods. The exposed em-



Fig. 3. (A) Hatching delay in zebrafish (*Danio rerio*) embryos exposed to 0, 20, 40, 60, 120, 240, and 360 mg/L of single-walled carbon nanotubes (SWCNTs) from 4 h postfertilization (hpf). Exposure to SWCNTs at 120 mg/L or greater induced significant hatching delay from 52 to 72 hpf. (B) Exposure to 120 mg/L of carbon black and double-walled carbon nanotubes (DWCNTs) did not induce any hatching delay. (C) Exposure to 240 mg/L of SWCNTs and DWCNTs induced a significant hatching delay from 56 to 72 hpf, but exposure to carbon black did not. All values are presented as the mean  $\pm$  standard error. An asterisk indicates a significant difference (analysis of variance, p < 0.05).

bryos were collected from the groups treated with 240 mg/L of SWCNTs. Using the myogenic regulatory factor MyoD as a marker, the myotomes were found to be well organized, with arrowhead-shaped alignment both in control (Supplementary Fig. 3A; SETAC Supplemental Data Archive, Item ETC-26-04-005; http://www.setacjournals.org) and SWCNT-exposed embryos (Supplementary Fig. 3B), and the notochords were found to be well organized, with a straight alignment in both control (Supplementary Fig. 3C) and SWCNT-exposed embryos in the dorsal view (Supplementary Fig. 3D). The development of primary sensory neurons, as determined using the proneural marker neurogenin-1 (ngn1), also was not affected, and ngn1 normally was expressed in neural plate regions as well as in rhombomeres (RBs) and dorsal root ganglion neurons. The subset of neurons in the peripheral cuticular and central nervous systems also were well developed, as determined using two zebrafish achaete-scute homologs, Zash 1a (Supplementary Fig. 3G and H) and Zash 1b (Supplementary Fig. 3I and J), as markers. At the protein level, the Zn5 antibody zebrafish neuron marker indicated that commissural neurons, axons, and cell bodies of the nVI abducens motor neurons at RB boundaries also were not significantly affected by SWCNT exposure (Supplementary Fig. 4; SETAC Supplemental Data Archive, Item ETC-26-04-005; http:// www.setacjournals.org). All the molecular and cellular experimental results provided further evidence that the exposed embryos developed normally. This series of experiments supports the notion that the chorion offers effective protection and acts

as the first barrier to agglomerates of SWCNTs (Supplementary Fig. 1B).

#### DISCUSSION

In the present study, we demonstrated that exposure to SWCNTs in the aquatic environment delayed hatching but did not influence embryonic development and survival of exposed zebrafish embryos. Similarly, exposure to DWCNTs induced a shorter hatching delay in zebrafish embryos, whereas exposure to carbon black did not cause any effect. Scanningelectron microscopic observation revealed that the chorion of zebrafish embryos acts as a strong protective barrier to prevent the passage of the large SWCNT clusters through the pores. We also observed that CNTs adhered easily to the chorion of the zebrafish embryos and the skin of larvae (data not shown), indicating that raw CNTs were biologically affinitive.

Previous studies have reported that zebrafish embryos displayed delayed hatching when exposed to the antirheumatic drug diclofenac [32]. Exposure to low-dose x-rays in zebrafish embryos also induced delayed hatching [33], indicating that hatching delay may be a kind of stress response in zebrafish embryos. The mechanism for the delayed hatching might be caused by a change in the elasticity of the chorion (because of the mechanical force posed by many SWCNT agglomerates adhering to its surface) or by interference, to some extent, from the SWCNT agglomerates with the digestive function of the chorionic hatching enzyme (influencing hatching time). Current membrane modeling and mechanical measurement



Fig. 4. The head-trunk angle (**A**) and the body length (**B**) of zebrafish (*Danio rerio*) embryos exposed to 240 mg/L of single-walled carbon nanotubes (SWCNTs), carbon black, and control. Exposure to 240 mg/L of SWCNTs and carbon black did not significantly affect the head-trunk angle of zebrafish embryos both at 24 and 48 h postfertilization (hpf; **A**) or the body length of zebrafish embryos at 48 hpf (**B**) compared to the control group.

techniques, however, are based on cell lines [34] and are not directly applicable to zebrafish embryos, and it was very difficult to monitor the mechanical properties of the chorion with the moving embryos inside.

The hatching delay observed during the exposure also might have been a result of hypoxia on exposure, because CNT agglomerates covered part of the chorion of the exposed embryos and this cover might interfere with the oxygen exchange. Previous work illustrated that hypoxia-exposed sheepshead minnow (*Cyprinodon variegatus*) embryos displayed delayed hatching and development in comparison to normoxic treatments [35]. Another set of experiments demonstrated that hyp-



Fig. 5. The appearance of pigment in the control (**A**) and zebrafish (*Danio rerio*) embryos exposed to 240 mg/L of single-walled carbon nanotubes (SWCNTs; **B**) at 42 h postfertilization. Embryos exposed to 240 mg/L of SWCNTs were more pigmented than control embryos (white arrows in **B**). Bar = 150  $\mu$ m.



Fig. 6. Blood vessel staining of control (**A**) and zebrafish (*Danio rerio*) embryos exposed to 240 mg/L of single-walled carbon nanotubes (SWCNTs; **B**) at 72 h postfertilization (hpf). Blood vessels were stained for endogenous alkaline phosphatase activity. Blood vessel formation in SWCNT-exposed embryos (**B**) was not significantly different from control embryos (**A**) at 72 hpf (see white dots in **A** and **B**). Bar = 150  $\mu$ m

oxia also slowed sand snail (*Polinices sordidus*) embryonic development before hatching, but development accelerated after hatching and, eventually, the embryos developed normally [36]. The embryos exposed to CNTs in the present study, however, did not show any slowed embryonic development. Furthermore, blood vessel staining indicated that the zebrafish circulatory system was not affected on exposure. Because the blood vessels supply tissues with oxygen, nutrients, hormones, and cellular and humoral factors, this result is quite consistent with other results that the embryonic development of the exposed embryos appeared to be unaffected during the time period tested.

The present results also provided important information regarding the particle size distribution of SWCNT agglomerates in the aquatic environment. Single-walled CNTs tended to form larger agglomerates in both freshwater and salt water at pH 11, which likely resulted from the fact that the SWCNTs were purified by acid leach and thermal oxidation and were covered with negatively charged carboxylic acid groups at the defect sites on their sidewalls. The particle size distribution results indicated that SWCNTs with negatively charged groups might prefer to form larger agglomerates under extremely basic conditions because of internal attractions. Generally speaking, this kind of basic situation is very rare in the environment, so changes in the SWCNT particle size distribution may not have great implications for the interaction of these structures with biological systems.

The hatching delay observed in zebrafish embryos in the present study might have been induced by the Ni and Co catalysts used in CNT synthesis. It is known that Ni and Co both inhibit hatching in zebrafish embryos, with Ni being more specific and potent. The nominal no-effect concentrations for hatching time were 40  $\mu$ g/L for Ni (NiSO<sub>4</sub>, 6 H<sub>2</sub>O) and 3,840  $\mu$ g/L for Co (CoCl<sub>2</sub>, 6 H<sub>2</sub>O) [37]. The raw SWCNTs used in the current experiments were formed with Co and Ni catalysts

at an atomic ratio of 3:1. The raw DWCNTs used in the current experiments were made with Mg<sub>1-x</sub>Co<sub>x</sub>O solid solution containing additions of Mo oxide; the Mg catalyst was removed during the synthetic process and only part of the Co catalyst remained [27]. It is likely that the remaining Co and Ni in the SWCNTs and, possibly, Co in the DWCNTs played an important role in the toxicity of these compounds, because these catalysts could not be totally removed. The remaining Co in DWCNT compounds is partially encapsulated in graphitic shells [38], whereas both Co and Ni occur in the SWCNT compounds and are not encapsulated in the nanotubes. This difference may be the reason why DWCNT compounds induced a hatching delay only at twice the SWCNT concentration. The purity of the SWCNTs was approximately 90 atom percent; therefore, the greatest Co and Ni concentrations would be approximately 7.5 and 2.5%, respectively, of the SWCNTs. At a SWCNT concentration of 120 mg/L, the greatest Co and Ni concentrations in the solution would be approximately 9 and 3 mg/L, respectively, if completely dissolved. The actual Co and Ni concentrations with respect to the embryos, however, possibly were much greater, because most of the SWCNT agglomerates clustered together on the chorions of the embryos. Although the concentrations of Co and Ni in the 120 mg/L of SWCNT solution likely were not high enough to affect hatching time, the concentrated Co and Ni associated with CNTs on the chorion could still induce significantly delayed hatching. It also is possible that the CNTs interact with the metal catalysts and strengthen their inhibitive effect, but this potential interaction has not been investigated in any study.

The manner in which raw SWCNTs and DWCNTs eliminated the normal function of PTU in solution also is hard to explain. This interference with PTU function might result from the direct adsorption of PTU into or, most likely, onto CNTs or from changes in PTU structure as a result of interactions with CNTs in solution. Interestingly, after centrifuge separation of SWCNTs and PTU from the incubation medium, pigment formation was still inhibited in embryos cultured in the supernatant fraction, but pigment formation occurred normally in embryos cultured with the resuspended pellet of SWCNTs plus additional PTU. This result indicated that SWCNTs did not remove PTU from the solution and that SWCNTs kept the ability to inhibit the effect of fresh PTU after incubation for 24 h (data not shown). Double-walled CNTs also inhibited PTU function in zebrafish embryos.

The PTU is used to delay pigment development in the embryos through inhibition of tyrosinase activity [30], and SWCNTs were reported to decrease tyrosine kinase activity in HEK293 cells [39]. Both PTU and SWCNTs are known to decrease tyrosinase and tyrosine kinase activities; however, their combined exposure did not inhibit pigment formation in zebrafish embryos in the current study but, instead, induced normal pigment development. The water in the CNT-dosed Petri dishes was much clearer than the water in the untreated controls, a result that also was observed in experiments with fullerene [8]. The CNTs, however, appear to be able to improve water clarity because of the adsorption of the waste in the aquatic environment instead of interference with the growth of beneficial bacteria [8]. These results implied that CNTs might be good remediation materials to adsorb toxicants from the environment [25].

The present study did not indicate that the SWCNTs are more toxic than the DWCNTs, both because their purities are hard to compare and because the catalysts are different. It is important to note that at present, most of the experiments reported in the literature have investigated the effects of CNTs mixed with catalysts and amorphous carbon, meaning that the tested effects actually were a combination effect, making it hard to evaluate the real toxicity of the CNTs alone. It also is important to note that the actual purity of CNTs often is far lower than that reported by manufacturers, and in reality, it is impossible to do elemental analysis for every batch of commercial CNTs products [40]. Finally, it should be noted that much of the current research seems to indicate that raw, uncoated CNTs are more toxic than purified CNTs [13].

Raw CNTs and associated nanomaterials might still pose a serious problem if they are released into the aquatic environment, although the present study demonstrated that exposure to the SWCNTs and DWCNTs did not induce any embryonic developmental malformations in zebrafish embryos. Any delay in the biological development of a species, however, is likely to have an effect, especially in the case of organisms that have short development periods or in which reproduction and development are synchronized with environmental cues, such as rainfall.

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