Aquatic toxicity assessment of single-walled carbon nanotubes using zebrafish embryos

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Abstract. Zebrafish embryos selected at the 64-cell stage were exposed to various concentrations of amide functionalized single-walled carbon nanotubes (SWCNTs) ranging from 1 to 10 μg/ml dissolved in 1% Pluronic F-68 (a cell culture grade surfactant), and the development of embryos was examined from 24 to 120 hours post fertilization (hpf). Incubation of embryos in 1% F-68 did not induce overt abnormal phenotype as compared to the wild-type; neither did it cause significant mortality during the exposure period. Generally, there was a slight developmental delay in larvae treated with SWCNTs of 5 μg/ml or above. Only larvae exposed to 5 μg/ml SWCNTs showed significantly reduced survival rates. About 50% of the embryos exposed to 5 μg/ml showed abnormal phenotypes at 24 hpf as compared to the control group. As development proceeds to 120 hpf, more embryos displayed defective morphology. A slight hatching delay was observed in embryos exposed to concentrations above 5 μg/ml. There was a general reduction of body axes, including narrowed somite and shortened yolk stalk. In addition, pigmentation in the ventral trunk area was less than that observed in control group. The body lengths of the exposed embryos were decreased significantly at 48 hpf (3.11 mm in control vs. 3.00 mm in SWCNTs-exposed embryos). However, exposure to SWCNTs did not affect the number of somites. Other features that were noticed in the SWCNTs-exposed embryos included edema and shrinkage and blebbing of the epidermal lining. Most of these observed phenotypes persisted from 48 hpf through 120 hpf. Overall, the aforementioned results indicate that soluble amide-functionalized SWCNTs are toxic to zebrafish embryos at a minimum concentration of 5 μg/ml.

1. Introduction
Carbon nanotubes (CNTs) are one of the most widely explored nanomaterials with great potential applications in electronics, chemistry and biomedicine [1]. With the increasing demand of the nanomaterial in industry, a growing public concern about their potential health risks is emerging dramatically [2,3]. CNTs seem to have unusual toxicity properties due to their structural resemblance to asbestos. Many studies have indicated that, in-vitro, CNTs exhibit substantial cytotoxicity, including the induction of oxidative stress, the inhibition of cellular proliferation and the induction of apoptosis/necrosis [5,6]. The toxic effects are also observed in exposed mice and rats [7,8]. Raw CNTs...
are extremely hydrophobic and can form large agglomerates in solutions. Functionalization of CNTs with different chemical molecules could enhance their solubility, but may thus result in differential cytotoxicity and biodistribution in the body than pristine ones. For example, intravenous administration of diethylenetriaminepentaacetic (DTPA) functionalized single-walled CNTs (SWCNTs) into mice followed by indium radioactivity tracing indicated that they are not retained in liver or spleen, but are rapidly cleared from systemic blood circulation through the renal excretion route [9]. Taken together, CNTs may produce toxicity and the toxic effects could be influenced by surface modifications.

CNTs may be possibly released into the environment during the processes of manufacturing, modification or use, which could lead to noxious interactions with biological systems to generate toxicity [10]. However, studies investigating the impact of CNTs on the environment are still limited. A recent study showed that raw SWCNTs induced slight hatching delay in zebrafish embryos [11], indicating that SWCNTs have potential toxic effects on aquatic environment. Zebrafish has been widely used in developmental and genetic research; their value in toxicology as well as drug discovery has also been recognized [12]. With the aid of large-scale and high-throughput screening method, zebrafish can provide a suitable vertebrate model for straightforward in vivo toxicity assessment and serve as an intermediate step between cell-based and mammalian testing. It has been reported that CNTs could be readily dispersed as an aqueous suspension by natural organic matter in the environment [12]. The dissolved CNTs may display an unexpected toxicity than pristine ones. Therefore, the aim of this study is to evaluate the potential toxic effect of soluble amide-functionalized SWCNTs on the development of zebrafish embryos.

2. Materials and methods

2.1. SWCNTs
Amide functionalized SWCNTs (Sigma) was used in this study. The SWCNTs are labelled with amide groups per 4 atoms. The average size of amide-SWCNTs is about 4-6 nm in diameter and 0.7-1 μm in length. Amide-SWCNTs powder are completely dissolved in 1% Pluronic F68 at a concentration of 1 mg/ml using sonicator for overnight and then ultrasonic cell disruptor for 5 min.

2.2. Zebrafish
Wild-type zebrafish (AB strain) embryos were raised and maintained at 28°C in a 14h:10h light:dark cycle according to standard laboratory conditions [14] and staged at 28°C according to Kimmel et al. [15]. At 2 hours post fertilization (hpf), the embryos were examined under a dissecting microscope, and those embryos that developed normally and reached the 64-cell stage were selected for subsequent experiments.

2.3. Embryo toxicity test
A stock solution of 2 mg/ml SWCNTs was prepared by dissolving SWCNTs in 2% Pluronic F-68 (Sigma). The selected embryos were finally exposed to 0, 1, 3, 5, 7.5 and 10 μg/ml of SWCNTs in 1% F-68. At least 10 embryos were randomly distributed into each well of the 24-well plate, 4 replicates were set up for each concentration. Exposure time was from 2 hpf to 120 hpf. The embryos were incubated at 28°C and the solutions were replaced every 48 hours. The embryos/larva were examined under a dissecting stereomicroscope at 12, 24, 48, 72, 96, 120 hpf for morphological abnormalities, and defective rates and survival rates were recorded for each replicate at all time points.

2.4. Whole-mount in situ hybridization (WISH)
myoD gene products were amplified from recombinant plasmids (pGEM-T easy) using PCR with SP6 and T7 primers. They were then used as template to synthesize digoxigenin (DIG)-labeled riboprobes using T7 RNA polymerases (DIG-labeling kit, Roche). The specificity of antisense probes was verified by running control experiments with sense probes. WISH experiments were performed as
described [16] on embryos fixed at 19 hpf. Hybridization was performed in 50% formamide and DIG-labeled probes were detected with anti-DIG alkaline phosphatase-coupled Fab fragments (1:5000) and NBT/BCIP (Roche). Embryos were cleared in graduate concentration of glycerol up to 50% before observations.

Figure 1. Morphological comparison of zebrafish embryos and larvae exposed to 1% F68 and 5 μg/ml of SWCNTs. A slight developmental delay was observed in SWCNTs treated larvae.

3. Results and discussion

3.1. Dose-dependent toxicity of SWCNTs on zebrafish embryos

Raw SWCNTs are highly insoluble. Functionalized SWCNTs, such as amide-CNTs, are more hydrophilic and dissolvable in 1% F68. Zebrafish embryos selected at the 64-cell stage were exposed to five concentrations of amide-SWCNTs (1, 3, 5, 7.5, 10 μg/ml), and the development of embryos was examined from 24 to 120 hours post fertilization (hpf) (Figure 1). Generally, there was a slight developmental delay in larvae treated with 5 μg/ml of SWCNTs or above. Incubation of embryos in 1% F68 did not cause significant mortality during the exposure period (Figure 2). As the concentration of SWCNTs increased, the survival rates of exposed embryos were significantly decreased. At 24 hpf, 95% of the embryos exposed to 5 μg/ml of SWCNTs were viable whereas embryos exposed to 7.5 and 10 μg/ml SWCNTs had survival rates of 83% and 62%, respectively (Figure 2). After five days of exposure, the survival rates of zebrafish larvae were significantly decreased to 75%, 20% and 42% in 5, 7.5 and 10 μg/ml of SWCNTs (p values were less than 0.05 at least vs. F68 control), respectively.

Figure 2. Survival rates of zebrafish larvae exposed to different concentrations of SWCNTs at different time points. Four replicate experiments were performed for each concentration. The data are presented as mean ± standard deviation. “*” and “**” represent p < 0.05 and p < 0.01, respectively, using the Student’s t test compared to the control group (F68).
Among the survived embryos, embryos exposed in 1% F68 control did not induce overt abnormal phenotype as compared to the wild-type. However, the percentage of embryos with normal phenotype was significantly lower in embryos exposed to $\geq 3 \mu g/ml$ of SWCNTs at 24 hpf ($p < 0.01$), and the normal rates continued to decrease at later post fertilization (Figure 3). Notably, the lowest normal phenotype rate occurred for embryos exposed to $5 \mu g/ml$ of SWCNTs for 120 hours. For instance, about 50% of the embryos exposed to $5 \mu g/ml$ showed abnormal phenotype at 24 hpf as compared to the control group. As development proceeds to 120 hpf, more embryos displayed defective morphology. This result could be explained by the higher mortality rates in embryos exposed to higher concentrations of SWCNTs (e.g., 7.5 and 10 $\mu g/ml$).

**Figure 3.** Normal rates of zebrafish larvae exposed to different concentrations of SWCNTs at different time points. “***” represents $p < 0.01$ compared to the control group (F68).

Previous studies have reported that exposure of zebrafish embryos to dispersed SWCNTs agglomerates in the aquatic environment delayed hatching but did not influence embryonic development and survival of exposed embryos [11]. Because the size of the pores on the embryo chorion was nanoscaled and the size of the SWCNTs agglomerates was microscaled, the authors suggested that the chorion of zebrafish embryos was an effective barrier for protection from SWCNTs agglomerates. In our study, the surfaces of SWCNTs were functionalized with amide groups to increase their hydrophilicity. We found that there was an increased dissolution for amide-SWCNTs in aquatic environment, which also correlates with an increased toxicity. Although a dose-dependent toxicity was observed on the survival of embryos exposed to 1-10 $\mu g/ml$ of SWCNTs, the maximal defective rate was observed when embryos were exposed to $5 \mu g/ml$ of SWCNTs for 120 hours. This is likely due to a reduced dissolution of SWCNTs at higher concentrations because agglomerates of larger sizes were found in solutions of concentration 7.5 $\mu g/ml$ or higher. Results from our study showed a much higher toxicity of SWCNTs than the previous one (toxic effects ranging from 5 to 10 $\mu g/ml$ in this study vs. minimal toxic effects ranging from 20 to 360 $\mu g/ml$ for the previous study), indicating that the toxic response of zebrafish embryos with chorion is dependent on the degree of dissolution of SWCNTs.

### 3.2. Phenotypes of zebrafish embryos exposed to SWCNTs

Under normal conditions (28.5°C in egg water), zebrafish embryos complete the rapid morphogenesis of primary organ systems and start to hatch out of the chorion from 48 hpf. We observed several developmental features, including somite formation, eye development, heart beat, blood circulation, pigmentation in head-body and in tail, pectoral fin formation, hatching and protruding mouth, of the
treated-embryos at different time points in comparison to the control group. Some of them are shown in Table 1. The abnormal phenotypes in embryos exposed to 5 μg/ml of SWCNTs were most obvious at 48 hpf.

Table 1. Observed phenotype changes in the developmental features of zebrafishs treated with 5 μg/ml of SWCNTs at different exposure times. The abnormal phenotypes in embryos were most obvious at 48 hpf. “*” indicates p < 0.05 from the control group (1% F68).

| Phenotype                  | Exposure Time | WT       | F68 (1%) | SWCNTs (5 μg/ml) |
|----------------------------|---------------|----------|----------|------------------|
| Hatching                   | 48 hpf        | 2/39 (5.1%) | 8/40 (20%) | 8/37 (21.6%)     |
|                            | 72 hpf        | 36/38 (94.7%) | 39/40 (97.5%) | 32/36 (88.9%)*  |
| Pigmentation               | 48 hpf        | 31/39 (79.5%) | 20/40 (50%)  | 10/37 (27.0%)*  |
|                            | 72 hpf        | 35/38 (92.1%) | 38/40 (95%)  | 34/36 (94.4%)    |
| Edema                      | 72 hpf        | 2/38 (5.3%)  | 1/40 (2.5%)  | 6/36 (16.7%)*    |
| Yolk stalk shrinkage       | 48 hpf        | 4/38 (10.5%)  | 6/40 (15%)   | 11/36 (30.6%)*   |

There was a general reduction of body axes, including narrowed somite and shortened yolk stalk (Figure 4). In addition, pigmentation in the ventral trunk area was less than that observed in control group (Figure 4). The body lengths of the exposed embryos were decreased significantly at 48 hpf (3.11 mm in control v.s. 3.00 mm in SWCNTs-exposed embryos) (Figure 5). However, exposure to SWCNTs did not affect the number of somites. Other features that were noticed in the SWCNTs-exposed embryos included shrinkage and blebbling of the epidermal lining, hatching delay and edema (Table 1). Most of these observed phenotypes persisted from 48 hpf through 120 hpf.

Figure 4. Comparison of morphology between the wild-type and SWCNTs-exposed embryos at 48 hpf. Note that the lengths of individual somites (indicated by the black bars) and of the yolk stalks (indicated by red lines with double arrows) were reduced in embryos exposed to 5 μg/ml of SWCNTs.

Although the body lengths and individual somite widths were reduced at 48 hpf, the analysis of the muscle differentiation markers myoD and myogenin by RT-PCR revealed that both genes were expressed in SWCNTs-exposed and control embryos at comparable levels (Figure 6). The whole mount in situ hybridization of embryos exposed to 5μg/ml of SWCNTs did not also indicate a clear difference in the organization and morphology of somites from controls, suggesting that SWCNTs did not affect the formation of somites.
Figure 5. The body lengths of zebrafish embryos exposed to 5 μg/ml of SWCNTs dissolved in 1% F68 at different time points. The body lengths were significantly reduced at 48 hpf in SWCNTs-exposed embryos as compared to the control group.

Figure 6. Expression of myoD and myogenin in control and SWCNTs-exposed embryos. (A) RT-PCR analysis of RNA isolated from embryos exposed to 1% F68 control or 5 μg/ml of SWCNTs at 48 hpf. EF, elongation factor, was amplified as a loading control. (B) Whole-mount in situ hybridization of embryos exposed to 1% F68 or 5 μg/ml of SWCNTs at 18 hpf.

4. Conclusion
In summary, we examined the effect of different concentrations of amide-SWCNTs on zebrafish embryos and found that solubilized SWCNTs could induce toxicity in zebrafish embryos by reducing their survival and normal morphology rates. The present study may help define a no observable effect
concentration for SWCNTs in the developing zebrafish embryos and thus provide a better understanding of their use in potential impacts on aquatic environment.

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