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Developmental toxicity of oxidized multi-walled carbon nanotubes on *Artemia salina* cysts and larvae: Uptake, accumulation, excretion and toxic responses☆

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ABSTRACT

Using *Artemia salina* (*A. salina*) cysts (capsulated and decapsulated) and larvae [instar I (0–24 h), II (24–48 h) and III (48–72 h)] as experimental models, developmental toxicity of oxidized multi-walled carbon nanotubes (O-MWCNTs) was evaluated. Results revealed that hatchability of capsulated and decapsulated cysts was significantly decreased ($p < 0.01$) following exposure to 600 mg/L for 36 h. Mortality rates were 33.8, 55.7 and 40.7% for instar I, II and III larvae in 600 mg/L. The EC_{50} values for swimming inhibition of instar I, II and III were 535, 385 and 472 mg/L, respectively. Instar II showed the greatest sensitivity to O-MWCNTs, and followed by instar III, instar I, decapsulated cysts and capsulated cysts. Effects on hatchability, mortality and swimming were accounted for O-MWCNTs rather than metal catalyst impurities. Body length was decreased with the concentrations increased from 0 to 600 mg/L. O-MWCNTs attached onto the cysts, gill and body surface, resulting in irreversible damages. Reactive oxygen species, malondialdehyde content, total antioxidant capacity and antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) activities were increased following exposure, indicating that the effects were related to oxidative stress. O-MWCNTs were ingested and distributed in phagocyte, lipid vesicle and intestine. Most of the accumulated O-MWCNTs were excreted by *A. salina* at 72 h, but some still remained in the organism. Data of uptake kinetics showed that O-MWCNTs contents in *A. salina* were gradually increased from 1 to 48 h and followed by rapidly decreased from 48 to 72 h with a range from 5.5 to 28.1 mg/g. These results so far indicate that O-MWCNTs have the potential to affect aquatic organisms when released into the marine ecosystems.

1. Introduction

Carbon nanotubes (CNTs) possess exceptional electrical, chemical, and physical properties, which enable them utilized in various applications, such as manufacturing (De Volder et al., 2013), medicine (Heister et al., 2013) and other industries (Baughman et al., 2002; Eatemadi et al., 2014). Moreover, advances in CNTs synthesis, purification and chemical modification make them suitable for more commercial applications. In this regard, multi-walled carbon nanotubes (MWCNTs) are the largest product segment, accounting for majority market volume. However, along with the rapid increase of related products and applications, CNTs are inevitably released into the aquatic environment from: 1) the direct entry to waterbodies from bioremediation; 2) rainwater and runoff from contaminated air and soils; 3) aerial and tyres deposition; 4) emissions from wastewater treatment plants (Boxall et al., 2007; Yang et al., 2008). Eventually, most of the CNTs enter into marine ecosystems (Klaine et al., 2008; Wei et al., 2010). Sun et al. (2016) predicted the environmental emissions of engineered nanomaterials using dynamic probabilistic modeling, and suggested that the environmental concentration of CNTs was at the “mg/kg” level. In addition, most of the CNTs-related products are “durable” products (such as tyres and other polymer composites) that have almost no CNTs release during use. A mass of CNTs will release when the products come to the disposal phase, resulting in a higher CNTs concentration in the future (Sun et al., 2016). Therefore, it is imperative to investigate the potential hazards posed by CNTs at high concentration prior to the wide use of them. Such knowledge will be useful in utilization of CNTs and managing risk in the future.

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In recent years, using aquatic invertebrates as models to assess the potential toxicity of nanomaterials has become prevalent (Ates et al., 2013; Petersen et al., 2010; Zhu et al., 2017). As an invertebrate zooplankton found in various seawater systems from lakes to oceans, *Artemia salina* (*A. salina*) plays a key role in the energy flow of food chain (Sorgeloos et al., 1986). The intrinsic characteristics and physiological features of *A. salina* make it as a suitable model for toxicology testing (Nunes et al., 2006; Rajabi et al., 2015). For example, its cysts are commercially available, and have been widely used in toxicology tests (Caldwell et al., 2003; Rotini et al., 2015). Moreover, many stages are divided along the development process of *A. salina*, and larvae in a uniform physiological condition can be hatched synchronously (Persoone et al., 1989; Sorgeloos et al., 1979). Recently, several studies have investigated the effects of nanomaterials on *A. salina* (Gambardella et al., 2014; Mesarič et al., 2015; Zhu et al., 2017). It is currently accepted that one of the major mechanisms for toxicity of nanomaterials is oxidative stress (Ates et al., 2013; Liu et al., 2012; Zhu et al., 2017). For example, Ates et al. (2013) investigated the impacts of Zn and ZnO nanoparticles on *A. salina*. They demonstrated that the nanoparticles showed significant toxicity to *A. salina* after exposure for 96 h, and the effects were related to oxidative stress. Furthermore, toxicity of graphene oxide (GO) to *A. salina* was evaluated in our previous study. Results showed that GO induced significant changes in hatchability, mortality, and morphological, ethological and physiological parameters (Zhu et al., 2017).

Accumulation and depuration behaviors of CNTs in organisms are critical factors for risk assessment, which impact the overall toxicity of CNTs (Edgington et al., 2013; Guo et al., 2013; Petersen et al., 2009, 2010). It was reported that MWCNTs could be significantly accumulated in the gut of *Daphnia magna*, but the accumulated MWCNTs were not entirely eliminated from the daphnia body (Petersen et al., 2009, 2010). Besides, we investigated the uptake and toxic effects of MWCNTs on *Saccharomyces cerevisiae*, and showed that MWCNTs were clearly visible in lysosome, vacuole, endosome, mitochondria, multivesicular body and perinuclear region (Zhu et al., 2016). Mesarič et al. (2015) evaluated the effects of three different carbon-based nanomaterials on *A. salina* larvae following exposure for 48 h. They suggested that the nanomaterials were ingested and concentrated in the gut, and attached onto the body surface of *A. salina* (Mesarič et al., 2015). To date, information about the accumulation, depuration and developmental toxicity of MWCNTs on *A. salina* is limited.

In the study, toxicity of oxidized MWCNTs (O-MWCNTs) to *A. salina* cysts (capsulated and decapsulated) and larvae (instar I, II and III) were evaluated. Based on previous data, it was hypothesized that: 1) hatchability, mortality, and ethological, morphological and biochemical parameters would be significantly changed; 2) effects would be accounted for O-MWCNTs rather than metal catalyst impurities, and were mediated by oxidative stress; 3) O-MWCNTs would be ingested, accumulated and excreted by *A. salina*. The study contributes to better understandings of the MWCNTs toxicity, and lay foundations for their future exploitation and application.

2. Materials and methods

2.1. Preparation and characterization of MWCNTs

MWCNTs were purchased from Chengdu Organic Chemicals Co., Ltd., Chinese Academy of Sciences (Chengdu, China), and the structural parameters are listed in Table S1. They were oxidized (O-MWCNTs) and labeled with fluorescein isothiocyanate (FITC-MWCNTs) according to the previous study (Zhu et al., 2016) (as described in the Supplementary material). To prepare the suspensions with nominal concentrations of 25, 50, 100, 200, 400 and 600 mg/L, O-MWCNTs were weighed on aluminum foil and placed in 1 L beakers containing 900 mL of filtered natural seawater (FNSW; 30‰ m/v; pH 8.6). The beakers were placed in an ice bath and then probe sonicated with an ultrasonic processor (Scientz-IID, China). The suspensions were sonicated for 1 h at 100 W using a 50% on/off cycle and left overnight at room temperature, and sonicated again.

O-MWCNTs were characterized by scanning electron microscope (SEM, Hitachi S-4800, Japan) and transmission electron microscope (TEM, JEM1200EX, Japan) with accelerating voltages of 15 kV and 100 kV, respectively. To estimate the hydrodynamic size distribution of O-MWCNTs in FNSW, a dynamic light scattering (DLS, Brookhaven BI200SM, USA) was used. An X-ray photoelectron spectroscopy (XPS; PHI-5600, Russia) was used to analyze elemental compositions and chemical states. Raman spectra were recorded on a HR800 spectrophotometer (Longjumeau Cedex, France) with an excitation wavelength of 785 nm. The contents of residual metal catalyst impurities in pristine and oxidized MWCNTs were measured using ICP-MS (Thermo Elemental X7).

2.2. Model organism

Commercially available dehydrated cysts (Tianjin, China) of *A. salina* were used and kept at 4 °C until required. Decapsulated cysts and instar I, II and III larvae were obtained as described previously (Zhu et al., 2017) (as described in the Supplementary material).

2.3. Hatching assay

Cysts were treated with O-MWCNTs suspensions (0, 25, 50, 100, 200, 400 and 600 mg/L) to study the effects on hatchability. In order to measure the contribution of metal catalyst impurities to the effect, cysts were cultivated in $FeCl_3$ solutions. The concentrations of Fe^{3+} were 0, 0.022, 0.044, 0.088, 0.176, 0.352, 0.528 mg/L, as the same amount of Fe^{3+} as 0, 25, 50, 100, 200, 400, 600 mg/L MWCNTs suspensions, respectively (Table S2). Hatching tests were carried out according to the previous study (Zhu et al., 2017).

A parallel set of experiments was performed to determine the O-MWCNTs settling behavior with *A. salina* cysts. Briefly, triplicate suspensions (0.5 mL) were sampled after exposure for 0, 6, 12, 18 and 24 h from each treatment. The samples were pipetted into a square quartz groove (side length: 5 mm; height: 1 mm) and then dried in vacuum at 80 °C. Contents of O-MWCNTs were quantitatively assessed using a HR800 Raman spectrophotometer (Longjumeau Cedex, France) with an excitation wavelength of 785 nm, and calculated by a standard curve.

2.4. Acute toxicity test

Acute toxicity test was performed by adding 10 larvae (instar I, II and III) to each well of 24-well plates. Each well contained 1 mL of O-MWCNTs suspensions (0, 25, 50, 100, 200, 400 and 600 mg/L) or $FeCl_3$ solutions. All plates were incubated with shaking at 28 °C under a 16:8 h light/dark management. Larvae were not fed during the test. After exposure for 24 h, the numbers of dead larvae were counted using a microscope (Olympus Optical Co., Ltd., Tokyo, Japan). All of the tests were carried out in octuplicate.

For each treatment, instar I, II and III larvae (approximately 1000) were also randomly added into beakers containing 100 mL O-MWCNTs suspensions or $FeCl_3$ solutions, and cultured at 28 °C with shaking. After exposure for 24 h, samples were randomly took and then immediately performed morphological and behavioral analysis, and ROS measurement. Samples for SEM and TEM analysis were fixed in 2.5% glutaraldehyde at 4 °C. Specimens for MDA