Evaluation of MWNT toxic effects on daphnia and zebrafish embryos

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Abstract. Organisms of daphnia (Daphnia magna) and zebrafish (Danio rerio) embryos were exposed to a range of different concentrations of COOH-functionalized MWCNT suspended in an aqueous solution of Tween 20. Immobilization of daphnia and growth retardation, inhibition and malformation of zebrafish embryos were the endpoints tested after 24 and 48 hours. Immobilization of daphnia could be observed from 3 to 16 ppm and an increasing mortality of zebrafish embryo was detected at all the concentration tested. To identify more subtle toxic effects, we took advantage of the extensive information available on the zebrafish genome and monitored by RT-PCR the expression patterns of different zebrafish genes that could act as toxicity bio-markers. At some of the concentrations tested, changes in the expression profiles of the genes examined were detected. Our results suggest that MWCNT could potentially represent a risk to human health and environment, therefore a wider range of concentrations and further testing of this molecules should be carried out to define possible limitations in their use.

1. Introduction
Carbon nanotubes are increasingly used in several industrial fields. Due to their unique electrical, mechanical and thermal properties they are currently employed in electronics, computer, biosensing and they have a potential application in the areas of food packaging for example as spoilage biosensors. In spite of the increasing popularity and vast use, their innocuity for human health still remains to be fully demonstrated. The objective of this work was to assess the potential toxic effects of multi walled carbon nanotubes (MWCNT) functionalized with a carboxylic group to facilitate dispersion in solutions. To achieve this aim we employed two well established ecotoxicological models: daphnias and zebrafish. In addition, zebrafish is an extensively used vertebrate model in developmental biology, pharmacology and medical science currently relying on a wide number of genetic tools including a nearly fully sequenced genome. In particular, in this study we employed zebrafish embryos that up to 48 h post hatching allow the performance of in vitro tests [1, 2]. The toxicity of carbon based nanomaterials using such model organisms has been investigated by several Authors and a number of studies have demonstrated some concerns especially in relation to C₆₀ fullerenes toxicity [3- 5]. The rationale of this work is to employ an acute toxicity test with daphnia as a first broad screening method and subsequently to detect more subtle effects of MWCNT on a model
vertebrate, by testing the differential expression of some selected toxicity biomarker genes on exposed zebrafish embryos.

2. Materials and methods

2.1. Pristine Test material
Commercial Catalytic Chemical Vapour Deposition Multi-Wall Carbon Nanotubes (CCVD MWCNTs), were used. The MWCNT characteristics were as follows: outer mean diameter, 10-15 nm; length, 0.1-10 µm; mean number of walls, 5 – 15; Carbon content above 90 wt% without detectable and free amorphous. To verify the presence of impurities, chemical analysis was carried out on the MWCNTs by Inductively Coupled Plasma Atomic Spectroscopy (ICP-AES). A termogravimetric analysis was also performed in diluted air in argon atmosphere using a TGA 92 16.18 SETARAM equipment applying a 2º C min⁻¹ ramp up to 1200ºC.

2.2. MWCNT functionalization
Carboxyl-functionalized CNTs were prepared by oxidation of MWCNT with HNO₃. MWCNT (300 mg) and HNO₃ (65%, 200 ml) were mixed in a 500 ml flask equipped with a condenser. The flask was then immersed in an ultrasonic bath under reflux at 60°C for 2 h. After cooling down to room temperature, the reaction mixture was filtered under vacuum through a polycarbonate filter of 0.2 µm pore size (Millipore). The solid was dispersed in 500 ml distilled water, sonicated in an ultrasound bath for 30 min and filtered again, repeating this washing until complete removal of the residual HNO₃. Finally, the CNT-CO₂H samples were oven-dried overnight at 60°C. Pristine and functionalized MWCNT were also characterised by Digital Instruments Multimode atomic force microscopy (AFM) using RTESP Rotated Tapping Mode Etched Silicon Probes at 300 KHz frequency by dispersing the sample of MWNTs in methanol and then depositing it on a special holder by a drop-drying method. The functionalized MWCNT were finally suspended in a solution of Tween 20 (160 mg/l) to aid dispersion and solubilisation. Toxicity tests were carried out on different concentrations of this MWCNT test solution.

2.3. Daphnia acute toxicity test
Daphnia magna neonates were exposed to four different concentrations of the MWCNT test solution: namely, 16; 12; 6 and 3 mg/l and to a control solution of Tween 20. Experiments were carried out in triplicate exposing 20 daphnias to each of the test solutions. Daphnia immobilization was assessed after 24 and 48 h.

2.4. Zebrafish embryos exposure
Fertilized embryos were obtained from adult WIK strain zebrafish (Danio rerio) bred and maintained in our laboratory following standard conditions [6]. Embryos at the same developmental stage (two hours post fertilization - hpf) were collected and rinsed in embryo water. Exposure experiments were carried by placing embryos at 2 hpf into individual wells of a 96-well plate containing 100 µl of the MWCNT test solution at concentrations of 12, 6 y 3 ppm. Embryos exposed to solutions of Tween 20 (160 mg/l) were used as controls. Exposure experiments were carried out in triplicate exposing 30 embryos to each of the concentrations tested. Morphological and developmental endpoints were observed under an optic microscope in treated embryos and controls 24 and 48 hpf. Embryos 48 hpf were then used for RNA extraction.

2.5. Expression analysis (quantitative real-time PCR)
RNA extraction was carried out in groups of 30 exposed embryos 48 hpf. RNA was extracted using TRizol (Sigma-Aldrich) following the instruction of the manufacturer. RNA quantity and quality was determined using a BioAnalyzer 2100 (Agilent). Reverse transcription reactions were carried out using
30-40 ng of total RNA using a TaqMan Reverse Transcriptase Reagents kit (Applied Biosystems). Reaction conditions were as follows: 25°C for 10 min, 48°C for 30 min and 95°C for 5 min.

Specific oligonucleotides were synthesized for the zebrafish genes Ahr2, iNOS and NMDAR using sequences stored in the NCBI and TIGR databases. Quantitative real time PCR (qRT-PCR) was performed using an ABI Prism 7000 Sequence Detection system (Applied Biosystems), with a SYBR Green PCR master mix (Applied Biosystems). 40 ng cDNA were used as a template. Reaction conditions were as follow: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation step was added at the end of the PCR. The β-actin gene was used as a constitutive control, to normalize all samples. The threshold cycles were calculated by the 7000 system software and expression levels of RNAs were calculated by the $2^{-\Delta\Deltact}$ method [7].

3. Results and discussion

3.1. Characterization of the MWCNT

The pristine MWCNT was subjected to termogravimetric and chemical analysis to assess its level of purity. TGA determination allowed detecting a 2% of catalyst impurities. Chemical analysis carried out by ICP-AES showed a content of 0.56% of Al and 0.93% of Fe and a percentage lower than 0.1% of Si and Mg. AFM analysis of the pristine material in comparison to the functionalized material showed that the functionalization improved MWCNT dispersion. (Figures 1a and 1b).

![Multimode atomic force microscopy (AFM) images of the MWCNT before (a) and after (b) functionalization with HNO$_3$.](image)

3.2. Daphnia acute toxicity

*Daphnia magna* individuals were exposed to the following concentrations of functionalized MWCNT: 16, 12, 6, and 3 mg/l. As shown in Figure 2, exposure to 16 mg/l of MWCNT caused the immobilization of 95% of the daphnias after 24 h and 100% after 48 h. On the other hand, exposure to 12 mg/l caused immobilization of 15 and 10% of the animals at 24 and 48 h, respectively. Exposure at 6 mg/l only caused immobilization in 10% of the daphnias after 48 h exposure. No immobilization was detected at exposure to concentration of 3 mg/l. Exposure to the control solution of Tween 20 did not seem to cause any detectable effect on the daphnias analysed.
3.3. Zebrafish embryos acute toxicity
Zebrafish embryos were exposed to the following concentrations of the MWCNT test solution 12, 6 and 3 mg/l and to the control solution of Tween 20. The control solution showed some degree of toxicity and caused the death of 20% of the individuals already at 24 hpf. Exposure to the MWCNT test solutions caused the death of 35 to 45%, slightly increasing with the increasing of the concentration. The vast majority of the deaths occurred at 24 hpf (see Figure 3).

3.4. Zebrafish embryos differential gene expression analysis
The differential expression of the following genes was tested by qRT-PCR: *Ahr2*, *iNOS* and *NMDAR*. These genes were selected as biomarkers on the basis of their function in biological processes related to the response to toxicant exposure and on the basis of previous transcription response analysis carried out with different toxicants [8]. In particular, *Ahr2* (Aryl hydrocarbon receptor 2) acts as an environmental checkpoint that senses exposure to environmental toxicants and responds by signalling
cell cycle inhibition; iNOS (inducible nitro oxide synthase) mediates tumoricidal and bactericidal activities of macrophages; NMDAR (N-methiyl-D-aspartic acid Receptor) acts as a neurotoxicity biomarker. No changes in gene expression were detected in the embryos treated with the solution of Tween 20; however some changes were detected at some of the exposure concentrations of the MWCNT solution. In particular, as shown in Table 1, an increase in expression was clearly detected for gene Ahr2 and this seemed to be dose responsive at the concentrations tested. NMDAR gene also showed an increase in the expression, but only at the highest concentration tested. These preliminary results indicate that MWCNT could potentially represent a risk to human health and environment.

Table 1. Differential expression analysis of some selected biomarker genes expressed from samples exposed to different concentration of MWCNT with respect to the control represented by the sample treated with a solution of Tween 20.

| EXPOSURE SOLUTIONS | GENES FOLD INDUCTION |
|---------------------|-----------------------|
|                     | Ahr2  | iNOS  | NMDAR |
| 3 mg/l              | 55    | 1     | 1     |
| 6 mg/l              | 150   | 1     | 1     |
| 12 mg/l             | 187   | 1.4   | 16    |

4. Conclusions
The results obtained indicate a possible toxic effect of MWCNT on both daphnia and zebrafish embryos for the concentrations tested. Studies carried out on SWCNT on zebrafish embryos [9] reported a delay in embryo development that the authors ascribed to the mixed effect of the CNT and of the catalyst contaminants. We do not exclude that the results obtained in the present study for both daphnia and zebrafish embryos are due to the action of MWCNTs, the catalyst contaminants as well as to the dispersant agent (in our case, Tween 20). It is indeed difficult to separate and clearly distinguish the single effect of each of these three factors; however, it should be noted that the biological application of MWCNTs is, in most cases, associated to some catalyst contaminant and to the presence of a dispersant agent. Therefore it is acceptable to consider the effect of these factors altogether. The results here presented indicate that probably the dispersant Tween 20 is not ideal for solubilising MWCNTs as it showed a degree of toxicity that was higher than expected. On the basis of the results obtained we are currently testing a wider range of dispersants, MWCNT concentrations and genes acting as toxicity biomarkers. The final aim of our research will be to define possible limitations in the use of MWCNT, especially in relation to a potential application in food packaging.

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