Potential artifacts and misinterpretations when evaluating the ecotoxicological effects of nanomaterials

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ABSTRACT

Engineered nanomaterials (ENMs) have significant commercial potential in a broad range of industries for consumer products as a result of their novel properties. However, these same properties may cause unexpected risks once ENMs are released into the environment either intentionally or unintentionally. Thus, standard methods are needed to accurately and reproducibly assess the potential risk of ENMs. One factor that limits the applicability of standard ecotoxicology test methods for use with ENMs is that the unique behaviors of ENMs may cause artifacts or misinterpretations in these tests as a result of their unique behaviors. We briefly discuss these artifacts and misinterpretations and provide an illustrative example.

Keywords: nanoparticle, nanoecotoxicology, standard test methods, artifacts, nanomaterials

1 INTRODUCTION

Nanotechnology promises exciting innovations in a broad range of fields, and nanomaterials have substantial potential for incorporation into consumer products. Nanomaterials are defined using the definition from the International Organization for Standardization (ISO): engineered nanomaterials (ENMs) are materials with any external dimension between 1 nm and 100 nm or having an internal surface structure in those dimensions [1, 2]; other agencies such as the FDA may not necessarily operate under this strict definition. One issue that has limited the commercialization of ENM-containing products is their potential impacts on humans and the environment. Standard methods are needed for assessing the potential risks of ENMs, but the behaviors of ENMs differ substantially from those of traditional environmental pollutants such as hydrophobic organic chemicals and inorganic pollutants such as lead. Moreover, a literature review of the nanotechnology environmental health and safety literature showed that uncertainty in the applicability of current standard test methods for use with ENMs is the most frequently cited source of uncertainty [3].

One of the substantial differences between the behaviors of traditional environmental pollutants and ENMs during ecotoxicology testing is that ENMs may cause artifacts and misinterpretations during many of these tests. While there have been numerous review articles on the ecotoxicity of ENMs in organisms [4-18], the potential experimental artifacts and misinterpretations during these tests have received substantially less attention. For example, artifacts have been previously observed in nanoecotoxicology testing as a result of an unintended byproduct produced during the ENM dispersion process [19, 20] and from ENM interference with an assay reagent [21-27]. Misinterpretations in nanoecotoxicology testing are also possible if the effect observed is mistakenly attributed to nanoparticles when dissolved ions are actually the cause of the toxic effect. In a recently submitted review article [28], we have systematically reviewed the potential artifacts and misinterpretations related to testing the potential ecotoxicological effects of ENMs. Potential artifacts were identified at every step of nanoecotoxicology testing: during the initial material synthesis or procurement and associated impurities [29-31], ENM storage [32-36], ENM dispersion [19, 20, 37, 38], unacknowledged indirect toxicity effects such as nutrient depletion [39-41], and during the toxicity assays [21-27, 42-44]. Test recommendations for how to avoid or minimize these artifacts and misinterpretations were also provided including a comprehensive list of potential control experiments and what they could test. In this proceedings paper, we will not reiterate the information from that review article, but rather briefly provide an example of an artifact observed as a result of coating desorption during storage which impacted the subsequent ecotoxicity measurement [36].

2 EXPERIMENTAL

The full experimental method is available in a previous publication [36]. Briefly, multiwall carbon nanotubes (MWCNTs) were treated with HNO_3/H_2SO_4 (v/v = 3:1), filtered, and rinsed with boiling water. These "3:1 MWCNTs" were then grafted with polyethyleneimine (PEI) as described previously [45]. MWCNTs were produced with positive (MWCNT-PEI), negative (MWCNT-PEI-Suc), or neutral (MWCNT-PEI-Ac) surface charges. These materials were thoroughly dialyzed and then stored at 4°C for several months.

Daphnia magna neonates (1 d to 2 d old) underwent immobilization tests at a range of concentrations (0 mg/L to 40 mg/L) for MWCNTs and for each of the three types of PEI-modified MWCNTs [46]. Five replicates of ten neonates in 20-mL vials were tested after 24 h and 48 h with 3:1 or PEI-coated MWCNTs spiked to artificial freshwater ($CaCl_2$ x $2H_2O$ 58.8 mg L^{-1}, $MgSO_4$ x 2 H_2O 24.7 mg L^{-1}, $NaHCO_3$ 13.0 mg L^{-1}, and KCl 1.2 mg L^{-1}; hardness $[Ca^{+2}]+[Mg^{+2}]$ = 0.5 mM). Several additional experiments were conducted to explore the potential for artifacts to influence the immobilization results. Solutions of each modified MWCNT at the highest concentrations tested were filtered using ashless Whatman cellulose filters (2.5 µm, grade 42) and *Daphnia* were exposed to the filtrate. The PEI polymer by itself was also tested; PEI-Suc and PEI-Ac were not tested as a result of experimental challenges associated with the synthesis, purification, and identification of these polymers in the absence of their covalent bonding to MWCNTs prior to dialysis. In addition, all three types of MWCNT-PEIs were dialyzed, and their toxicity tested immediately after dialysis. The percentages of *Daphnia* immobilized after 24 h and 48 h of exposure were plotted against test concentrations and the data analyzed by statistical probit method (BioStat 2009, AnalystSoft) to calculate EC_{50} values (i.e., the concentration at which 50 % of the *Daphnia* become immobilized) and their 95 % confidence limits.

3 RESULTS AND DISCUSSION

One important unexpected finding of this study was that, while the filtrate from the MWCNT-PEI-Ac and MWCNT-PEI-Suc did not cause immobilization, the filtrate from the MWCNT-PEIs caused 18% immobilization. This suggested that the PEI itself may exert a significant toxic effect on the neonates. When the PEI by itself was tested, the 24 h EC_{50} value was 19.3 mg/L which is within the range tested for the MWCNTs. When the MWCNTs were dialyzed immediately before the immobilization experiment, the toxicity for the MWCNT-PEI were significantly reduced (see Figure 1) while those for the MWCNT-PEI-Suc and MWCNT-PEI-Ac were unchanged. These results suggest that desorption of the PEI occurred to the MWCNT-PEI during storage which caused an overestimation of the MWCNT-PEI toxic effects. This type of result prevents direct assignment of the toxicity to the nanomaterial. This example demonstrates the value of conducting a filtrate-only control experiment to investigate potential toxic effects from compounds released from the ENMs.

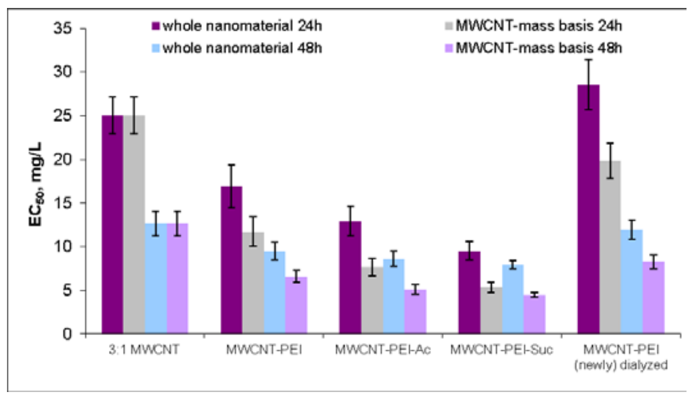

Figure 1: EC_{50} values for *Daphnia magna* exposed to regular and PEI modified MWCNTs. Five replicates of ten neonates were tested per concentration, and five to seven concentrations were tested for each type of MWCNT. Values are given after 24 and 48 h for the whole mass of the MWCNT with the PEI coating as indicated by "whole nanomaterial," and on the basis of the MWCNT core by itself as indicated by "MWCNT-mass basis." Values provided for the MWCNTs after they were recently dialyzed are marked "(newly) dialyzed." Error bars represent the 95 % confidence intervals. This figure is modified and reprinted with permission from [36] copyright (2011) American Chemical Society.

ACKNOWLEDGEMENTS

Certain commercial products or equipment is described in this paper in order to specify adequately the experimental

procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that it is necessarily the best available for the purpose.

The study was supported in part by federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. T.B. Henry acknowledges EU FP7-NMP-2013-LARGE-7 Project Number 604602. J.C. White also acknowledges USDA AFRI 2011-67006-30181.

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