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Inactivation of *Escherichia coli* planktonic cells by multi-walled carbon nanotubes in suspensions: Effect of surface functionalization coupled with medium nutrition level

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HIGHLIGHTS

- MWCNTs were functionalized by acid treatment and adsorption of organic matter.
- Bacterial toxicity of MWCNTs in suspensions was assayed with *E. coli* planktonic cultures.
- MWCNT surface modifications significantly exerted impacts on the dispersivity of MWCNTs.
- Inactivation of planktonic cells was both MWCNT functionalization- and medium nutrition-dependent.
- Dispersity alone may not be a proper index to estimate CNT bacterial toxicity on suspended cells.

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ABSTRACT

While earlier studies have identified the antibacterial activity of carbon nanotubes (CNTs) and proposed that cell membrane damage by direct contact with CNTs is likely the main toxicity mechanism, the relative importance of chemical versus physical properties of CNTs in controlling their bacterial cytotoxicity is understudied. Given that CNT is commonly modified via acid treatment to enhance its dispersivity and surface chemistry, in this study commercially available multi-walled carbon nanotubes (MWCNTs) with high purity were processed carefully by acid reflux, resulting in differences in surface charge of MWCNTs without altering their physical properties. The surface condition of MWCNTs was also modified by adsorption of organic matter to compare bacterial toxicity of functionalized and non-functionalized MWCNTs in suspensions. Results show that although overall electrostatic repulsion and steric obstruction resulted from surface modifications led to elevated dispersivity of MWCNTs and mitigated toxicity on planktonic *Escherichia coli* cultures, no correlation between the dispersivity and bacterial toxicity of MWCNTs was observed, suggesting that dispersity alone may not be a proper index to estimate the CNT antibacterial effect on planktonic cells in the aqueous phase. In addition, viability recovery of MWCNT-treated cells was observed to be nutrition level-dependent, implying that availability of proper nutrients may be another important factor to be considered when assessing the ecotoxicity of CNTs in the aquatic system.

1. Introduction

Nanotechnology – the study and application of materials with characteristic dimension between 1 and 100 nm – has rapidly evolved into one of the most important disciplines over the past decades, as materials at this scale can display novel and unexpected physicochemical, optoelectronic, and thermal properties that differ significantly from their respective bulk counterparts, thereby providing a fascinating basis for innovation in a wide array of industrial sectors [17]. However, such unique size-dependent properties of nanomaterials that have made them useful in manufacturing different products may also give them potential biologically-disruptive effects on living organisms, and hence considering that (i) current rapid large-scale development of nanotechnology seems to inevitably increase the likelihood of releasing these engineered nanomaterials (ENMs) into the environment and (ii) the behavior of ENMs in the environment is still not fully understood [5], concerns of potential ecological health risks associated with their use have been raised [28]. As a result, many studies to date have focused

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on human health and ecological implications of ENMs to promote sustainable use of these novel materials [33,26].

One of the main aspects in the environmental impact of ENMs is to understand the interaction of ENMs and microorganisms, as microbes form the foundation of most ecosystems and are the key players in catalyzing a number of essential biogeochemical processes, providing crucial environmental services [41]. In fact, it has been reported that ENM-microbe interactions are mutual: ENMs have direct impacts on the physiology and survival of bacteria, and in turn, the structure thus stability of ENMs is affected by the activity of bacteria [16,8]. This further emphasizes that a better understanding of the relationship between ENM properties and responses of microbes at molecular, cellular and community levels is crucial to more accurately predict and interpret the ultimate fate and toxic effect of ENMs in the environment [38].

In the case of CNTs, Kang et al. [12] reported the first direct evidence on the strong antibacterial activity of CNTs using *Escherichia coli* K12 (ATCC 25404) as the model organism. Because the single-walled carbon nanotubes (SWCNTs) used by the authors were highly purified, their data clearly demonstrated that severe damage of cell membrane resulting from direct physical contact with SWCNT aggregates was the likely mechanism responsible for the leakage of intracellular contents that eventually led to cell death. Subsequent work revealed that SWCNTs in general were more toxic than MWCNTs due to the relatively shorter length that increased the contact opportunity for cell-CNT interactions [13]. Time dependence of bactericidal effect of CNTs was reported as well [15]. More interestingly, electronic structure-dependent antibacterial activities displayed by SWCNTs confirmed the role of oxidative stress involved in the CNT cytotoxicity mechanism [39], and measurements on the cytoplasmic membrane fluidity of bacteria indicated physiologically adaptive responses of bacteria to CNTs [45].

Despite these important findings, contradictory results regarding the effect and pathway of CNT toxicity toward bacteria are also reported in the literature. For example, compared to the monocultures of Gram-negative strains that were assayed under the same experimental conditions, Gram-positive strains experienced little, higher, or even comparable cytotoxicity in the study of Kang et al. [15], Liu et al. [22], and Arias and Yang [2], respectively. Moreover, in contrast to the observation made by Vecitis et al. [39] showing that cellular oxidative stress was the primary SWCNT cytotoxicity mechanism, Liu et al. [22] demonstrated that the physical puncture of SWCNTs on bacteria was the major cause responsible for cell death. While future research that aims to provide mechanistic explanations for these variations is warranted, all available data to date have consistently pointed to that CNT cytotoxicity is initiated upon direct contact of CNTs and cells, which strongly implies that dispersity of CNTs may play an important role in determining the overall antibacterial efficiency of CNTs, as highly dispersed CNTs in general would facilitate greater opportunities to come in contact with bacterial cells.

The degree to which CNTs are dispersed and stabilized in aqueous media is known to be predominantly controlled by chemical modifications of their surface, through either covalent or non-covalent functionalization [3,36]. It is found that CNTs can be readily dispersed as an aqueous suspension in solution containing natural organic matter and remained stable for a prolonged period (e.g., on the order of a month), resulting in elevated mobility of CNTs even in the porous environment [40]. However, investigations into the effect of CNT aggregation and dispersibility on bacterial toxicity so far have not reached a definitive conclusion [1,37,42], which may have been resulted from differences in the exposure condition, medium chemistry, solution ionic strength, agitation intensity, or/and CNT physicochemical property between research studies. This lack of agreement not only highlights the complexity and difficulty in probing CNT-cytotoxicity, but also points out the need for future designs of laboratory experiments that can properly decouple the effects of CNT-surface chemistry from CNT-physical characteristics.

Given that both SWCNTs and MWCNTs display approximately identical toxicity pathways but the properties of MWCNTs are inherently easier to modify and control [30], in this study we conducted laboratory experiments using highly-pure and well-characterized MWCNTs as model CNTs and *E. coli* as a model organism to investigate the effect of surface functionalization and medium nutrition level on CNTs' bacterial cytotoxicity. MWCNTs were functionalized carefully by acid treatment (a covalent modification) and adsorption of organic matter (a non-covalent modification) including humic acid (HA) and dimethyl phthalate (DEP) onto MWCNTs. In particular, we focused on the elucidation of the link between dispersivity and antibacterial activity of CNTs on suspended bacterial cells.

2. Materials and methods

2.1. Preparation and characterization of MWCNTs

MWCNTs, produced by chemical vapor deposition, of 20–40 nm in diameter, 5–15 μm in length, and ≥95% purity were purchased from Conyuan Biochemical Technology Inc., Taiwan (MWCNTs-20). The percentages of amorphous carbon and residual metal catalyst in as-grown MWCNTs (A-MWCNTs) were reported by the manufacturer to be <3 wt% and ≤0.2 wt%, respectively. Previous studies have shown that metal toxicity was not an important mechanism of bacterial cytotoxicity of SWCNTs and MWCNTs containing residual metal catalyst as high as 0.8 wt% (mainly cobalt) and 6.7 wt% (mainly iron), respectively [14]. A-MWCNTs were dispersed in 3M HNO_3 and refluxed at 120 °C for 3 h to introduce functional groups on A-MWCNT surface. The A-MWCNTs and HNO_3 were then separated by centrifugation at 25–30 °C and 8000 rpm for 10 min. The HNO_3-treated A-MWCNTs, designated as H-MWCNTs, were washed by deionized water (DI water, resistance >18 MΩ) repeatedly until the solution pH stabilized at 6.

Characterizations of MWCNTs are detailed in Supporting Information.

2.2. Adsorption experiment

Batch adsorption experiments were performed by adding 0.02 g of MWCNTs into 100 mL of an HA (Sigma-Aldrich) or DEP (Sigma-Aldrich) solution that had been adjusted to pH 6 at different concentrations (HA: 0.5–12 mg C/L; DEP: 10–160 mg/L) in 125-mL amber glass bottles. A stock HA solution was prepared by dissolving HA in DI water and filtering the solution through a 0.45 μm cellulose nitrate membrane filter, resulting in a concentration determined by a TOC analyzer (multi N/C 2100S, Analytik Jena) equal to 130 mg C/L. The MWCNTs and HA or DEP mixtures were sonicated for 30 min, followed by agitation in a shaker at 25 °C and 150 rpm for 1 h. After equilibration, syringe-filtered (0.45 μm pore-size Teflon membranes) aliquots were measured by TOC and HPLC (LC-6AD, Shimadzu) to determine the level of HA or DEP remained in the solution phase. All the adsorption experiments were conducted at least twice.

2.3. MWCNT dispersion & stabilization experiment

MWCNT suspensions were prepared following the procedures of adsorption experiments, but after 1 h vigorous agitation, the amber glass bottles were kept static for 7 days. During that, the unsettled supernatant was carefully collected by a syringe, which was then filtered using a Whatman Grade 41 filter paper (20–25 μm nominal