Toxicity of Multi-walled Carbon Nanotubes in rat and human intestinal cell models

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Abstract

Multi-walled carbon nanotubes (MWCNTs) are multiple one-atom thick sheets of carbon. MWCNTs can improve the mechanical, electrical and thermal conductivity of many products so they are used in a wide variety of applications. The manufacture and use of MWCNTs may result in their release into the environment. Exposure to MWCNTs may occur following inhalation and ingestion of these materials. The purpose of this study was to assess the in vitro toxicity of MWCNTs with outside diameter (OD) of <8nm, 13-18nm, >50nm, and 20-30 nm and two functionalized MWCNTs (-OH, -COOH) with OD 20-30 nm in a rat (IEC-6 cells) and human intestinal cell models. MWCNTs suspended in Dulbecco's media, 10% fetal bovine serum and 0.1% pluronic were probe sonicated for 15 minutes before dosing. IEC-6 cells were plated in 96-well plate with 60K cells/well for 24h. Media with 10% fetal bovine serum was the negative control and Triton X-100 (0.3%) was the positive control. Cells were then exposed to the MWCNTs at various concentrations (0.3-300 µg/mL) for 24 h. Following incubation, the cells were washed with media and the cytotoxicity was assessed using a cell-permeant dye, Calcein AM. This is a non-fluorescent compound that is converted to the green-fluorescent Calcein in viable cells upon acetoxymethyl ester hydrolysis by intracellular esterases. Lethal concentration$_{50}$ of the MWCNTs were determined: <8 and 20-30 nm OH, 35 µg/mL; 20-30 nm, 50 µg/mL; 20-30 nm COOH, 80 µg/mL; 13-18 nm, 105 µg/mL; 50 nm, 300 µg/mL Cell viability measured in the human intestinal model using the colorimetric MTT assay showed no cytotoxicity by the MWCNTs. For the rat intestinal model, size of MWCNT has a role in their cytotoxic effect.

Introduction

- MWCNTs are allotropes of carbon. They contain multiple concentric graphene layers and are formed by multiple one-atom thick sheets of carbon.
- MWCNTs can improve mechanical, electrical and thermal conductivity in products so they are used in wide variety of applications including ceramics, aerospace, drug delivery and others.
- Studies have shown a potential toxicity induced by MWCNTs following inhalation or ingestion. MWCNTs can enter the gastrointestinal tract following ingestion or inhalation followed by mucociliary clearance from the pulmonary tract.
- Short-term exposure of MWCNTs after inhalation or instillation in rodents have shown adverse health effects such as genotoxicity, fibrosis, and pulmonary inflammation. The gastrointestinal tract toxicity of MWCNTs is not well known.

Objective

The objective of this study was to examine the cytotoxicity of four pristine MWCNTs with outside diameter of <8 nm, 13-18 nm, >50 nm, and 20-30 nm and two functionalized (-OH, -COOH) with outside diameter 20-30 nm in a rat 2-D and human 3-D model system of the small intestine.

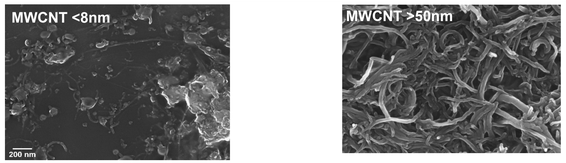
Materials and Methods

• Rat small intestine epithelial cells (IEC-6) were from Advanced Tissue Culture Collection (Manassas, VA) and four pristine (<8nm, 13-18nm, >50nm, and 20-30nm) and two functionalized (-OH, -COOH) 20-30nm multi-walled carbon nanotubes (MWCNTs) were from Cheap Tubes (Grafton, VT).
• IEC-6 cells were seeded at approximately 6 x 10^4 cells/well in a 96-well microtiter plate and placed in an incubator (37ºC, 5% CO_2, 95% RH) for 24 h before nanoparticle exposure.
• MWCNTs were suspended (300 µg/mL) in cell culture media with 10% fetal bovine serum and 0.1% Pluronic. The nanoparticles were dispersed with a probe sonicator (15 min. @ 4.5W). Optimum dispersion was determined using a Malvern Zetasizer.
• IEC-6 cells were treated with MWCNTs (0 to 300 µg/mL) and incubated for 24 h at 37ºC, 5% CO_2, 95% RH. The positive control was Triton X-100 (0.3%) and negative control was media with 0.1% Pluronic.
• At 24 h, the media was removed, and the cells were washed with media. Cell viability was assessed using Calcein AM, a non-fluorescent compound. Calcein AM is converted to the green-fluorescent Calcein in viable cells upon acetoxymethyl ester hydrolysis by intracellular esterases.

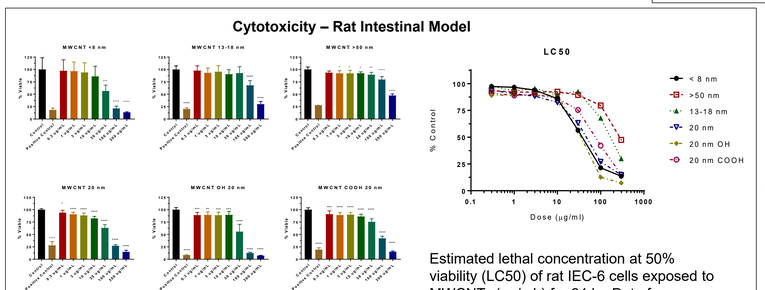
Materials and Methods (cont'd)

• Microscopic images of MWCNT <8nm and >50nm were taken using a confocal microscope. Cells were stained with 3 µM Calcein AM, and 6uM Propidium Iodide (Molecular Probes) in serum-free media for 20 min prior to imaging. Cell-free fluorescence readings of MWCNTs was also performed. Fluorescence of the MWCNTs was not detected.
• Cytotoxicity of MWCNTs in human intestinal model was measured. A human small intestinal 3-D model, Epiintestinal™ (SMI-100), was purchased from MatTek Corp. (Ashland, MA). The tissue is cultured with an air-liquid interface in 6-well plates with media below the tissue.
• The tissue was treated with MWCNTs (0 to 30 µg/mL) for 24h. Triton X-100 (0.3%) was the positive control. After 24h, the tissue was washed with PBS and viability was assessed using the colorimetric MTT assay.
• Toxicity of MWCNTs at the genomic level was also assessed. Total RNA was extracted from IEC-6 cells using a mirVana™ RNA extraction kit (Life Technologies. Grand Island, NY) following manufacturer's protocol. In short, cells were lysed and RNA was extracted using acid phenol-chloroform, then precipitated with ethanol. The RNA was purified through glass fiber columns, and RNA integrity was assessed using RNA 6000 LabChip kit using a 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).
• The gene expression change was analyzed by using PrepX RNA-seq for Illumina (Takara, Mountain View, CA). The data was processed using Partek Flow and Ingenuity Pathway Analysis (IPA).

Results

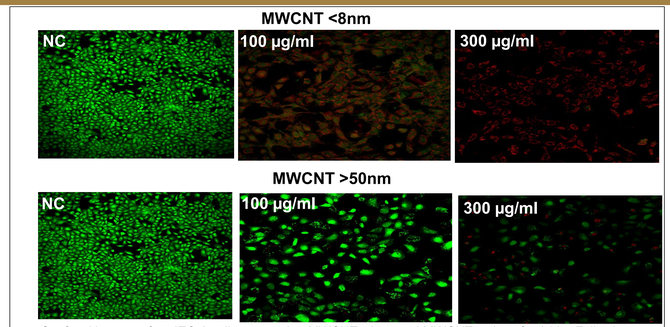


Scanning electron micrographs of < 8 nm and 50 nm MWCNTs

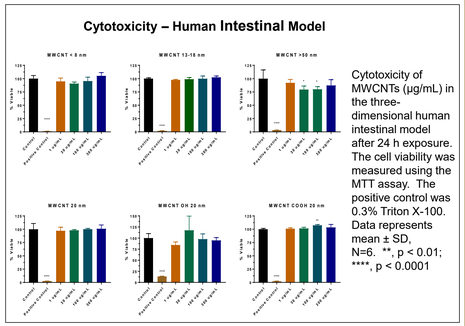


Cytotoxicity of MWCNTs (µg/mL) in rat IEC-6 cells after 24 h exposure assessed using the Calcein AM assay. The positive control was 0.3% Triton X-100. Data represents mean ± SD, N=6-12. *, p < 0.05; **, p < 0.01; ***, p <0.001; ****, p < 0.0001

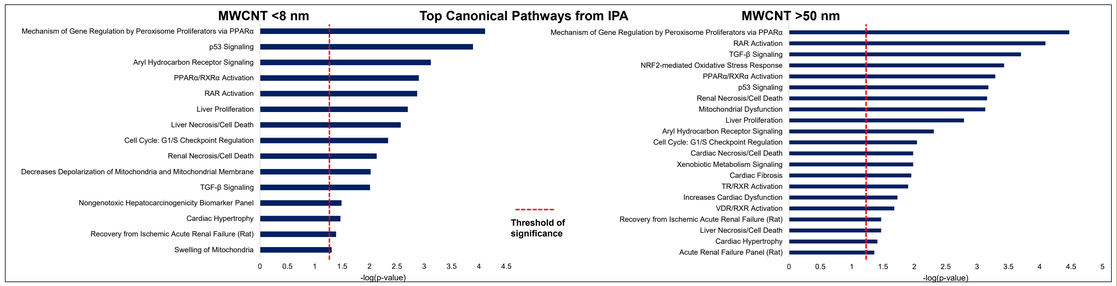
Estimated lethal concentration at 50% viability (LC50) of rat IEC-6 cells exposed to MWCNTs (µg/mL) for 24 h. Data from Calcein AM assay. Estimated LC50 was < 8 and 20 nm OH, 35 µg/mL; 20 nm, 50 µg/ml; 20 nm COOH, 80 µg/ml, 13-18 nm, 105 µg/mL; 50 nm, 300 µg/mL.



Confocal images of rat IEC-6 cells exposed to MWCNT <8nm and MWCNT >50nm for 24 h. Following incubation, the cells were stained with 3 µM Calcein AM (live cell) and 6 µM Propidium Iodide (dead cell).



Cytotoxicity of MWCNTs (µg/mL) in the three-dimensional human intestinal model after 24 h exposure. The cell viability was measured using the MTT assay. The positive control was 0.3% Triton X-100. Data represents mean ± SD, N=6. **, p < 0.01; ****, p < 0.0001



	Concentrations					
Nano particle	***0.3 µg/mL***	***1 µg/mL***	***3 µg/mL***	***10 µg/mL***	***30 µg/mL***	***Sum of DEGs***
MWCNT <8nm	3730	2745	2897	3102	163	12637
MWCNT >50nm	1068	2322	2163	2090	2862	10505

Table 1. Number of Differentially Expressed Genes (DEGs) following 24-h exposure to <8 and >50 nm MWCNTs in rat IEC-6 cells

Summary

• The rank order of the cytotoxic potential (lowest to highest) of the MWCNTs based on the Calcein AM assay shows that 50 nm < 13-18 nm < 20 nm COOH < 20 nm < 8 nm and 20 nm OH
• Calcein AM assay shows a reduction in fluorescence intensity starting at 30 µg/mL for MWCNT <8 nm and at 300 µg/mL for MWCNT >50 nm, indicating a reduction in cell viability. The fluorescence images of Calcein AM correspond to the cytotoxicity results.
• Similar study was performed on human small intestine (EpiIntestinal™) 3-D tissue model using MTT assay but no cytotoxicity was observed with any particle size. The rat 2-D intestinal model is more sensitive to the MWCNTs than the human 3-D intestinal model.
• The low number of DEGs with the <8 nm MWCNTs at 30 µg/mL may be due to the higher toxicity at the concentration.
• The outside diameter of the MWCNTs appears to be a factor in the cytotoxicity in IEC-6 cells, a 2-D cellular model.
• There are similar genomic responses between the two MWCNTs that suggests involvement of PPARα activation and p53 signaling.

Note: The contents of this poster do not necessarily represent U.S. EPA policy. The authors thank Sheau-Fung Thai, Carlton Jones, Hongzu Ren, Beena Vallanat, Mark Surette and Kim Rogers of the US EPA for technical assistance. This project was supported in part by an appointment to the Internship/Research Participation Program at the U.S. EPA administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and EPA.