Cytotoxicity of carbon nanotube variants: A comparative in vitro exposure study with A549 epithelial and J774 macrophage cells

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Abstract

While production of engineered carbon nanotubes (CNTs) has escalated in recent years, knowledge of risk associated with exposure to these materials remains unclear. We report on the cytotoxicity of four CNT variants in human lung epithelial cells (A549) and murine macrophages (J774). Morphology, metal content, aggregation/agglomeration state, pore volume, surface area and modifications were determined for the pristine and oxidized single-walled (SW) and multi-walled (MW) CNTs. Cytotoxicity was evaluated by cellular ATP content, BrdU incorporation, lactate dehydrogenase (LDH) release, and CellTiter-Blue (CTB) reduction assays. All CNTs were more cytotoxic than respirable TiO₂ and SiO₂ reference particles. Oxidation of CNTs removed most metallic impurities but introduced surface polar functionalities. Although slopes of fold changes for cytotoxicity endpoints were steeper with J774 compared to A549 cells, CNT cytotoxicity ranking in both cell types was assay-dependent. Based on CTB reduction and BrdU incorporation, the cytotoxicity of the polar oxidized CNTs was higher compared to the pristine CNTs. In contrast, pristine CNTs were more cytotoxic than oxidized CNTs when assessed for cellular ATP and LDH. Correlation analyses between CNTs’ physico-chemical properties and average relative potency revealed the impact of metal content and surface area on the potency values estimated using ATP and LDH assays, while surface polarity affected the potency values estimated from CTB and BrdU assays. We show that in order to reliably estimate the risk posed by these materials, in vitro toxicity assessment of CNTs should be conducted with well characterized materials, in multiple cellular models using several cytotoxicity assays that report on distinct cellular processes.

Introduction

Due to unique physico-chemical properties, engineered nanomaterials are increasingly applied in a number of areas, including chemistry, physics, electronics, materials science and biomedical sciences, thus drawing investments from governments and industries. Carbon nanotubes (CNTs) belong to a class of emerging engineered nanomaterials and are considered the material of the twenty-first century because of their attractive physical and chemical properties, and potential applications in advanced engineering, medical technologies, as well as in consumer products. These materials are also found in certain combustion emissions and occur naturally (Velasco-Santos et al., 2003). For instance, they are formed during burning of methane, propane and natural gas (Murr et al., 2004). CNTs can be either single-walled (SWCNT) or multi-walled (MWCNT), based on the number of layers constituting their structures.

Environmental and occupational exposure to nanomaterials may increase substantially in the near future as a result of their widespread use. The toxicity associated with CNTs can be ascribed to their shape and size, surface properties, presence of impurities (such as metals, amorphous carbon and fullerens) and the route of exposure (Di et al., 2009; Lam et al., 2006; Lee et al., 2010; Mu et al., 2008; Park et al., 2009; Pillai et al., 2007; Walker et al., 2009; Zhu et al., 2009). Health consequences of exposure to these materials are not clear (Stern & McNeil, 2008) due to the lack of reliable toxicity data (Huczko & Lange, 2001; Kislin et al., 2007; Monteiro-Riviere & Inman, 2006; Oberdorster, 2010; Shvedova et al., 2005; Warheit et al., 2004). There are also conflicting findings on toxicity characteristics of neutral and charged CNTs (Gys et al., 2008, 2010; Mingwu et al., 2009). Many of the published reports lack information on the CNT production method, presence of functionalities and impurities such as amorphous carbon and metal catalysts, and overall physico-chemical characteristics. It is critical to conduct comparative toxicity studies on well-characterized CNT batches in order to gain insight into the determinants of toxicity. In vitro cytotoxicity assays are particularly useful for screening CNT panels for biological reactivity as they are faster and less costly than in vivo animal exposure studies.

The aim of this study was to assess the impact of oxidation and removal of metallic contaminants from both single-walled
and multi-walled CNTs on their cytotoxicity to human lung epithelial cells A549 and murine monocytic cells J774. Inhalation is a likely route of exposure to CNTs with penetration of the aerosol in the deep portion of the lungs. Thus, alveolar lung epithelial cells and macrophages are potential early targets of CNTs. Both the A549 and J774 cell lines have been widely used by investigators as in vitro models including cytotoxicity assessments. We compared the effects of four variants of CNTs, pristine and oxidized, single-walled and multi-walled CNTs, in four cytotoxicity assays: the extracellular release of cytoplasmic LDH, the decrease of cellular ATP, the alteration of oxidation-reduction balance reflected in reduced rate of resazurin reduction, and inhibition of cellular proliferation determined by BrdU incorporation in DNA.

Experimental

Materials and reagents

Single-walled CNTs were synthesized by a pulsed laser-oven method using cobalt and nickel as catalysts (Kingston et al., 2004). Multi-walled CNTs were purchased from Sun Nanotech (China; >80 % purity; 10–30 nm in diameter), and were produced by chemical vapour deposition using iron as catalyst. Standard reference materials (SRMs), respirable cristobalite; SiO2 (SRM-1648a) and titanium dioxide; TiO2 (SRM-154b), were utilized as micron-sized reference particles. They were obtained from NIST (Gaithersburg, MD). The SRM particle characteristics are described in the NIST certificates.

Human lung epithelial (A549) and murine macrophage (J774A.1) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell culture medium and fetal bovine serum (FBS) were from HyClone (Logan, UT), and gentamicin was obtained from Sigma-Aldrich. Both A549 and J774 cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM, Fisher Scientific, Nepean, ON, Canada) supplemented with FBS (10% v/v) and gentamicin (50 μg/mL). Cells were propagated in 75 cm² tissue culture flasks (Corning Inc., Corning, NY) at 37 °C in 5% CO₂ and 95% relative humidity.

CellTiter-Blue (CTB) and LDH (CytoTox-96) cytotoxicity assay kits were from Promega Corporation (Madison, WI). ATP assay kits were purchased from Lonza Corporation (Rockland, ME), whereas the BrdU assay kits were obtained from Roche Diagnostics (Laval, QC, Canada).

Oxidation of CNTs

CNTs were oxidized mainly to remove catalytic impurities by the following procedure. Two hundred milligram of either single-walled or multi-walled CNTs were dispersed in a mixture of sulphuric acid (96%) and nitric acid (70%) at a ratio of 3:1 (v/v) and sonicated for 2 h. The CNT mixture was then diluted with deionized water and recovered on 0.45-μm filters. CNTs were washed with water until the washings showed a pH >5. The oxidized CNTs were dispersed by sonication of the filters in deionized water for 30 min, and clarified by centrifugation at 12 000 × g for 20 min. The precipitate containing impurities such as amorphous carbon was discarded. The CNTs were recovered from the clarified suspension on 0.45-μm filters, and the solid material was dried in an oven at 60 °C. Materials thus collected are referred to as oxidized CNTs.

Physico–chemical characterization of CNTs

Surface area and pore volume

Surface area and pore volume of CNTs were calculated from nitrogen adsorption and desorption measurements at 77 K using a Coulter Omnisorp 100 gas analyzer (Das et al., 2007). Before exposure to nitrogen, all CNT samples were degassed at 80 °C under high vacuum (10⁻³ Torr). Specific surface area (S_{BET}) was determined from the linear part of the BET plot (P/P₀ = 0.05–0.15).

Electron microscopy of CNTs

Samples of the CNT variants were dispersed in methanol by sonication, dropped onto a holey carbon grid and examined using a Philips CM12 transmission electron microscope (TEM; Oregon) operating at 80 kV (Kingston et al., 2004; Salam, 2006).

Metal analysis

Metal contents of pristine and oxidized CNTs were analyzed using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES, Varian Vista-Pro, Mulgrave, Australia) on the acid-digested samples (n = 3). In brief, carbon nanotube samples were digested in 50% HNO₃ for 8 h at 80 °C. These nanotubes were filtered out (0.22-μm filter) and metal contents in filtrates were analyzed by ICP-AES (Kim & Jo, 2006). A reagent blank was analyzed by ICP-AES and the blank values were subtracted from the sample analysis results to obtain the actual metal concentrations in these samples.

Infrared spectroscopy

Infrared analyses of CNT variants were performed on an ABB Bomem MB Series Fourier Transformed Infrared Spectrometer (FTIR; Victoria, Australia) using KBr pellets (Das et al., 2007).

Quantitation of surface COOH group

The amount of surface carboxylic groups present in oxidized samples was quantified by titration against NaOH (Eitan et al., 2003).

Particle size measurement in liquid media by dynamic light scattering

The hydrodynamic diameter of the CNT variants was determined by dynamic light scattering (DLS). CNTs were suspended in DMEM with fetal bovine serum (5% v/v) at 1 mg/ml concentration, vortexed for 10 s followed by sonication for 15 min. After sonication, the CNTs were diluted to 0.05 mg/ml concentrations and sonicated for 15 min followed by size measurements using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) employing a nominal 5 mW HeNe laser operating at a 633 nm wavelength; the scattered light was detected at 173°. The refractive index (1.33) and the viscosity (0.89) of ultrapure water at 25 °C was used in the data analysis performed in automatic mode using the instrument software (DTS 6.0). All measurements were performed 15 times to obtain an average particle size measurement. The particle size was reported as hydrodynamic diameter obtained as intensity distribution by Cumulant analysis.

Cell culture

Transmission electron microscopy of cells exposed to CNTs

All four types of CNT variants were suspended in particle buffer (containing 25 μg/mL Tween-80, 0.19% NaCl in water) at a concentration of 3 mg/mL and vortexed. The CNTs were dispersed using a Dounce glass-glass homogenizer (Nadeau & Lane, 1988), transferred to eppendorf tubes sealed with screw cap, sonicated for 20 min in an ultrasonic water bath and sterilized for 30 min in a water bath at 56 °C. A working suspension of
particles was prepared in serum-free medium and was sonicated before adding to the cell monolayers. For TEM imaging, A549 and J774 cells were seeded at $2 \times 10^4$ and $4 \times 10^4$ cells/cm² in six-well plates (9.6 cm²) in 2 mL/well of complete media and exposed to CNTs (30 μg/cm² dose in 2 mL of serum-free medium) for 24 h (37 °C, 5% CO₂). The cells were then fixed by replacing cell culture medium with 2.5 mL/well of glutaraldehyde solution (1.6% v/v) prepared in PBS (1X). The cells were stored in the fixative at 4 °C until further processing. Cells were then washed twice with 100 mM cacodylate buffer for 10 min followed by post-fixing with 1% osmium tetroxide in 100 mM cacodylate (pH 7.4), dehydrated in a series of graded alcohols at ascending concentration, infiltrated with Spurr’s epoxy resin and embedded within resin-filled gelatin Beem capsule molds. Ultrathin sections were post-stained with uranyl acetate and lead citrate, and examined with a Jeol 1230 TEM equipped with a CCD camera and AMT imaging software.

In vitro exposure to CNTs for cytotoxicity assays

A549 cells are a Type II lung epithelial cell line, whereas J774 cells are blood monocytes/macrophages. J774 cells will be referred to in the text hereafter as macrophages. Monolayers of A549 cells were trypsinized and washed with complete cell culture medium. On the other hand, J774 cells were scraped, and filtered with 100 μm cell strainers (Corning Inc., Corning, NY) prior to centrifugation (300 x g, 10 min at room temperature). Cell pellets were suspended in fresh medium and the cell density was adjusted to $2 \times 10^5$ cells/mL for A549 and $4 \times 10^4$ cells/mL for J774 cells for all assays with the exception of the BrdU assay, in which case the A549 and J774 cells were adjusted to $2 \times 10^5$ and $4 \times 10^5$ cells/mL, respectively. The cells were seeded in 96-well cell culture plates at 0.1 mL/well of complete cell culture medium and the plates were incubated at 37 °C for 24 h. Pristine and oxidized single and multi-walled CNTs suspensions were added to the wells at doses of 0, 3, 10, 30 and 100 μg/cm² in a final volume of 200 μL/well containing 5% FBS. The cell culture plates were incubated again for 24 h before the assays were carried out. Each exposure experiment was repeated at least three times and within each experiment exposures were done in triplicates for each experimental condition, and the mean results are presented.

Cytotoxicity measurements

Sequence of assays

After 24 h of exposure to particles, 100 μL of supernatant was transferred into a clear 96-well plate and clarified at $300 \times g$ for 5 min (room temperature); 25 μL was used for LDH assay, 75 μL was frozen. The CTB reagent, 50 μL prepared in culture medium (40% v/v) was then added to the remaining 100 μL of culture medium and the cells were incubated (5% CO₂, 37 °C) for 2 h. Aliquots (20 μL) were taken for measurement of CTB reduction at 10 min and 2 h as described below. The cell culture supernatant was discarded by aspiration and the cells were lysed with 200 μL of lysis buffer (100 mM MgCl₂ and 0.025% Triton X-100 in PBS) at room temperature, for 10 min. The lysate was recovered in clean plates and clarified by centrifugation as above; 25 μL of lysate was used for LDH measurement, 50 μL was used for ATP measurement, and 100 μL was frozen. The BrdU assay was conducted in separate cell culture plates as described below. For all assays, supernatants and cell lysates were clarified by centrifugation to prevent interference of CNTs in the assays.

CTB assay

In the CTB assay, viable cells reduce a non-fluorescent redox dye resazurin (dark blue in color) to a fluorescent reaction product resorufin (pink in color); non-viable cells lose metabolic capacity to convert the indicator dye. Mitochondrial, cytosolic and microsomal enzymes have been implicated in the reduction of resazurin (Gonzalez & Tarloff, 2001). For measurement of CTB reduction, 20 μL of supernatant was collected after 10 min incubation (baseline), and 20 μL was collected after 120 min. The aliquots were transferred into clean plates containing 80 μL of serum-free medium per well, shaken at 350 rpm for 30 s on a circular plate shaker, and clarified by centrifugation in a Sorval Legend RT at 300 x g (accelerated to 1500 rpm and then turned off). Fluorescence of the diluted supernatants was measured by top reading at λEx = 540 and λEm = 600 nm (Synergy 2, BioTek, Winooski, VT). CTB reduction is calculated by fluorescence at 120 min minus fluorescence at 10 min.

LDH assay

The CytoTox 96 colorimetric assay quantitates the activity of cytosolic lactate dehydrogenase (LDH) released extracellularly during phagocytosis, membrane damage, and upon cell lysis. The enzymatic activity released in the cell culture supernatants and recovered in the lysis buffer was measured with a coupled enzymatic reaction. NADH produced from NAD⁺ during oxidation of lactate to pyruvate is consumed simultaneously in a diaphorase-catalyzed reduction of tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, generating a soluble red formazan which is detected by absorbance at 490 nm. For the assay of released LDH, 25 μL of the cell supernatants was combined with 25 μL of cell culture medium and 50 μL of LDH substrate from the assay kit. Absorbance at 490 nm (Synergy 2) was measured after 20 min and 40 min of incubation in dark. For the assay of cellular LDH, 25 μL aliquots of the cell lysates was combined with 25 μL of lysis buffer and 50 μL of substrate from the LDH assay kit. Absorbance at 490 nm was measured immediately after 10 min of incubation in dark. The release of LDH was calculated as fraction of total LDH, which is LDH activity in supernatant divided by total LDH activity recovered in supernatant and cell lysate.

ATP assay

The ViaLight Plus is a bioluminescent assay for measurement of cellular ATP. Cell injury leading to mitochondrial injury results in a decrease of cellular ATP essential for cell metabolism. In the presence of ATP and oxygen, the firefly enzyme luciferase oxidises luciferin to oxyluciferin with the emission of photons. Chemiluminescence in the assay is proportional to the concentration of ATP in the cell lysate. The ATP reagent was prepared 15 min prior to conducting the assay by mixing ATP monitoring reagent and the assay buffer provided in the kit. Fifty microliters of the cell lysate reagent provided in the assay kit was added to each well of a chemiluminescence compatible white-walled 96-well plate, followed by 100 μL of freshly prepared ATP reagent. To the above mixture, 50 μL of cell lysate was added and incubated for 2 min in dark, and luminescence was measured (Synergy 2) (Crouch et al., 1993) with 1 s integrated readings per well.

BrdU assay

The BrdU Cell Proliferation ELISA is an enzyme immunoassay based on the incorporation of the thymidine analog 5-bromo-2’-deoxyuridine (BrdU) during DNA synthesis in proliferating cells, binding the incorporated BrdU with a peroxidase-coupled anti-BrdU, and detection by chemiluminescence from H₂O₂ oxidation.
of luminol (Porstmann et al., 1985). Cells were grown in black-walled 96-well plates and exposed to CNTs for 24 h as described above. The BrdU labelling medium (10 μM BrdU) was added to each well and the cells were incubated for 4 h in 5% CO₂ at 37°C. The labelling medium was then removed and the plates were dried at 60°C for 1 h, and stored at -40°C until use. The cell monolayers were fixed and denatured with the 200 μL of the fixation-denaturant reagent for 30 min and incubated with anti-BrdU antibody for 2 h at room temperature. The wells were washed three times with 150 μL of PBS containing 0.01% Tween-80, and the substrate provided in the BrdU ELISA kit was added. The plates were covered either with aluminium foil and were shaken for 4 min. Chemiluminescence was measured (Synergy 2) with 1 s integrated readings per well.

**Statistical analysis and potency estimation**

Cytotoxicity data for CNTs were analyzed using three-way ANOVA with CNT (SWCNT, MWCNT), Mod (pristine, oxidized) and Dose (0, 3, 10, 30, 100 μg/cm²) as factors, and by two-way ANOVA for the reference particles with PM (TiO₂, SiO₂) and Dose (0, 3, 10, 30, 100 μg/cm²) as factors. Statistical significance was considered for α = 0.05. Potency (β) is defined as the slope of the dose-response curve, according to:

\[
\text{Fold} - \text{effect} = (\text{Dose} + 1)^{\beta}
\]

where fold-effect (FE) represents the ratio of the value for a given dose of particles to the value in zero dose control cells (Vincent et al., 1997). Associations between physico–chemical properties and potency (β) values were analyzed using Pearson Product Moment Correlation Analysis. Statistical analyses were carried out using SigmaStat software (Jandel Scientific Inc., San Rafael, CA).

**Results**

**Physico–chemical characterization of CNTs**

**Surface area and pore volume**

Pristine MWCNTs exhibited higher surface area and pore volume values than pristine SWCNTs (Table 1). Surface characteristics of both MWCNTs and SWCNTs changed after the oxidation process resulting in lower porosity and surface area in the dry state.

**Transmission electron microscopy of CNTs**

Transmission electron microscopy of pristine SWCNTs revealed highly entangled tubes with diameters of about 5 nm (Figure 1). Similarly, the pristine MWCNTs were entangled tubes with diameters of about 20 nm. Although it is difficult to measure the length of the CNTs from TEM images, lengths of some CNT tubes were measured to be at least 1 μm for SWCNTs and ≥500 nm for MWCNTs. Both the pristine SWCNTs and pristine MWCNTs contained amorphous carbonaceous impurities along with the nanotube structures. Oxidation altered the surface morphology of both SWCNT and MWCNT, while shortening of tubes was noticed with oxidation of MWCNTs.

**Oxygen containing group (–COOH) analyses**

Titration of surface ‘‘oxygen-containing’’ groups such as carboxylic acid moieties against sodium hydroxide standard solutions revealed that oxidized CNTs, especially the single-walled ones contained higher levels of acidic functional groups on their surfaces. For instance, the levels of ‘‘oxygen-containing’’ groups were assessed to be at 0.93 mmol/g (pristine SWCNT), 1.21 mmol/g (pristine MWCNT), 4.83 mmol/g (oxidized SWCNT) and 4.20 mmol/g (oxidized MWCNT).

**Dispersion and size distribution of CNTs**

The suspensions of all four variants of CNTs in particle buffer were stable for up to 1 h (Figure 3). Nevertheless, the suspensions of pristine CNTs were less homogeneous and appeared to have a higher tendency to flocculate. In order to assess the size of the CNTs in liquid media, we carried out DLS analysis in the cell culture media with 5% FBS approximating the cell exposure conditions. The results are presented in Table 3. Hydrodynamic sizes of the CNTs were variable with high degree of agglomeration/aggregation at the concentration of 0.05 mg/mL. The MWCNT-O showed the least agglomeration/aggregation as indicated by low polydispersity index (PDI). In addition, the oxidized CNTs showed smaller hydrodynamic size than their respective pristine counterparts.

**Metal analysis**

The amounts of metallic impurities associated with each variant of CNTs were measured using ICP-AES (Table 2). The Co, Ni, Fe and Mo contents in pristine SWCNTs decreased with oxidation. Similarly, Fe and Ni contents decreased after oxidation of MWCNT. The Co and Mo contents of both pristine and oxidized MWCNTs were below the method detection limit, <0.002%.

**Infrared spectroscopy**

FTIR spectra of pristine and oxidized CNTs (Figure 2) revealed peaks at 3500 cm⁻¹ in both pristine and oxidized CNT spectra, which are attributed to the stretching vibrations of −OH groups. Two small peaks at 2854 and 2924 cm⁻¹ that are clearly observed with pristine and oxidized MWCNTs and less pronounced in SWCNTs are assigned to C–H stretching vibrations. An intense peak at 1730 cm⁻¹ and a broad peak at 1130 cm⁻¹ in the spectra of oxidized SWCNT and MWCNT are assigned to C=O and C–O stretching vibrations of the carboxylic acid (–COOH) groups. This 1730 cm⁻¹ peak is absent in pristine SWCNT and MWCNT spectra. Aromatic C=C stretching vibrations are observed at 1645 cm⁻¹ and at 1450 cm⁻¹ in all four spectra with comparable intensities.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface area, m²/g</th>
<th>Pore volume, cc/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pristine SWCNT</td>
<td>89</td>
<td>0.30</td>
</tr>
<tr>
<td>Oxidized SWCNT</td>
<td>21</td>
<td>0.11</td>
</tr>
<tr>
<td>Pristine MWCNT</td>
<td>106</td>
<td>0.44</td>
</tr>
<tr>
<td>Oxidized MWCNT</td>
<td>23</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 1. Surface area and volume of pristine and oxidized single- and multi-walled carbon nanotubes.
bundles clearly contained well-isolated fibers (Figure 1), and it is unlikely that agglomeration totally masked the nanotopology and surface properties of the CNTs.

**Cytotoxicity assays**

Dose-dependent release of cellular LDH was observed for J774 cells (Figure 5A) and A549 cells (Figure 5B). Pristine CNTs were clearly more potent than oxidized CNTs with respect to alteration of cytoplasmic membrane integrity (three-way ANOVA; J774, Dose × Mod × CNT, \( p = 0.008 \); A549, Dose × CNT, \( p < 0.001 \)). The CNTs decreased the rate of reduction of CTB in a dose-dependent manner for both J774 cells (Figure 6A; Dose, \( p < 0.001 \)) and A549 cells (Figure 6B; Dose, \( p < 0.001 \)). In contrast to the effect on LDH release, oxidized CNTs were more potent than pristine CNTs with respect to alteration of some components of the oxidation-reduction balance (Figure 6A, B; Mod, \( p < 0.001 \)). The decrease in cellular ATP correlated with effects in the LDH assay, with higher potency of pristine CNTs in J774 cells (Figure 7A;
Dose, \( p < 0.001; \) Mod, \( p = 0.031 \) and A549 cells (Figure 7B; Dose \( \times \) Mod, \( p = 0.003 \)). Effects of CNT exposures on BrdU incorporation, an indicator of cell proliferation, correlated with effects observed in the CTB assay, although effects of modification (factor Mod) was not statistically significant (Figure 8A and B). Interestingly, SWCNTs were generally more potent than MWCNTs in the BrdU assay (Figure 8A; J774, CNT, \( p = 0.036 \)), while MWCNTs appeared more potent than SWCNTs in the CTB assay (Figure 6A; J774, CNT, \( p = 0.054 \)).
662 m²/g has been reported in literature (Birch et al., 2013). Although, there is evidence for increase in surface area of CNTs after oxidation, the combination of the above mentioned reasons may contribute to the decrease in surface area as observed in our case. It is likely that surface area and pore size of CNTs in dry form do not predict surface area and pore size in the hydrated state in culture medium and once internalized by the cells.

The analyses of metal contents in CNTs revealed that the oxidation process led to remarkable decrease in Co and Ni contents, catalysts used in the production of pristine SWCNTs and similarly in Fe and Ni amounts in pristine MWCNTs. In addition,
our results suggested that oxidation process also removed co-contaminants Fe and Mo that were originally present in pristine SWCNTs (Figure 1). It is clear from this, that the acid treatment employed to oxidize CNTs removed to a good extent, the metal catalysts. Fourier transform infrared (FTIR) analyses results indicated the formation of surface polar groups as a result of oxidation of CNTs. The stretching vibration peaks of C=O group at 1730 cm$^{-1}$ and that of C–O group at 1130 cm$^{-1}$ in the spectrum of oxidized MWCNT and SWCNT (Figure 2) are not seen in their pristine counterparts confirming the formation of carboxylic acid (–COOH) groups in oxidized CNTs (Kathi & Rhee, 2008). Our analysis results on the levels of ‘‘oxygen-containing’’ groups on the surface of CNTs indicated that although pristine CNTs contain these polar functional groups due to surface defects during production, oxidation can increase the levels of surface functionalities. The process of oxidation can induce the formation of these polar functionalities not only at the tube openings, but also on the side walls with groups such as phenol, lactone, –COOH, ether and quinone on the sp$^3$ carbons (Wang et al., 2009). These results support our data obtained by FTIR analysis.

In general, pristine CNTs with sp$^2$ hybridized carbon-containing structures are relatively hydrophobic and can agglomerate in aqueous solutions. In contrast, following oxidation of the CNTs, creation of polar groups and more surface defects can contribute to increased hydrogen bonding and thus to enhanced solubility of the oxidized CNTs. In our study, all CNTs were dispersed with 0.025% Tween-80 in a glass-glass Dounce homogenizer followed by sonication. A visibly homogeneous and stable suspension of CNTs in serum-free medium was obtained after the sonication process for 20 min (Figure 3). Oxidized CNTs were comparably more dispersed while their pristine counterparts tended to flocculate with time, which is consistent with an enhanced solubility due to surface polar
Our DLS analysis of the CNTs revealed that all the materials remained in agglomerated/aggregated state in the cell exposure media used. It is well known that the CNTs interact with components (e.g. ions, proteins, etc.) of the dispersing medium, thereby changing the hydrodynamic diameters. The higher degree of agglomeration in the case of pristine CNTs may be attributed to their surface chemistry (oxidized CNTs, with surface COOH groups, is relatively less hydrophobic than pristine) and their interactions with the components of DMEM. The PDI values for all except MWCNT-O were \(0.5\) indicating that the hydrodynamic size values should be interpreted with caution. These observations illustrate the drawbacks of assessing size distribution of CNTs in aqueous suspensions by DLS, and warrant different approaches for such analysis (e.g. TEM). It should be noted further that DLS technique is applicable only to spherical particles. So, the measurement of size of CNTs, with high aspect ratios and coiled structures, using this technique is debatable. However, despite the limitations of the technique, the shape of CNT agglomerates can be assumed spherical in the liquid media and the measured sizes of the agglomerates can be utilized as a good approximation. Others have arrived at similar conclusion as well (Alpatova et al., 2010; Wang et al., 2012).

The lung epithelial A549 cells and macrophage J774 cells were chosen to address the pulmonary toxicity of CNT variants. It should be noted that despite being a lung adenocarcinoma-derived cell line, A549 cells possess numerous cellular markers of the Type II pulmonary epithelium (cell morphology, lamellar bodies, P450 enzyme activity, Surfactant Protein-C Expression) and serve as an useful model for in vitro studies of the biological effect of particulate matter including nanomaterials. Similarly, J774 peripheral blood macrophages are well characterized cellular model to study particle effects in vitro and are phagocytic, secrete macrophage-specific cytokines, express Fc receptors and synthesize lysozyme.

Thus, we chose A549 lung epithelial cells and J774 mouse macrophages since they are well characterized stable cell lines suitable for investigating in vitro cytotoxic potential of particles as mentioned above, and our intention here was to be able to...
assess whether these CNT variants were cytotoxic irrespective of the type and origin of the cell line.

Presumably, for A549 cells exposed to CNTs, reactions at the cell surface may be dominant, while for J774 cells, CNTs can be internalized by phagocytosis (Figure 4). It has been shown previously that bio-corona formation on the CNT surface may lead to the modification of the biological identity of CNTs thereby influencing their uptake by the cells (Lanone et al., 2013; Saptarshi et al., 2013).

The individual cellular bioassays revealed differences in cytotoxic potentials of these CNTs. The J774 macrophages were generally more responsive than the A549 epithelial cells (Figures 5–8), consistent with differences in the nature of the interaction of the cells with CNTs. Notwithstanding differences in the mode of interaction, very similar patterns of cytotoxicity were observed between the two cell types. In both cell types, oxidized CNTs were more potent than pristine CNTs with respect to alteration of CTB reduction and BrdU incorporation, while pristine CNTs were more potent than oxidized CNTs with respect to decrease of cellular ATP and increased leakage of cytoplasmic LDH (Table 4).

It is well established that redox cycling metals can reduce oxygen and generate reactive oxygen species such as superoxide anion, singlet oxygen, hydrogen peroxide, lipid hydroperoxides and hydroxyl radical. For example, Kagan et al. (2006) have shown that non-purified iron-rich CNTs cause oxidative stress in macrophages. However, we have previously observed an increase in the production of o-tyrosine, a marker of reactive oxygen species formation, in J774 cells exposed to oxidized CNTs, but not after exposure to the pristine CNTs (Kumarathasan et al., 2012). Since oxidized CNTs contained relatively less metal contaminants than pristine CNTs, it appears that the increased surface polarity of oxidized CNTs can also contribute to redox coupling reactions enabling an oxidative stress. We postulate that high cytotoxic potency for the oxidized CNT variants in the CTB and BrdU assays ($\Delta\text{CTB/BrdU}$) may be related to oxidative stress.
Figure 8. BrdU incorporation. All data are normalized relative to control (0 μg/cm²) and are presented as mean ± SE (n = 5–6 experiments). (A) J774 cells. Three-way ANOVA, Dose, p < 0.001; Mod, p = 0.079; CNT, p = 0.036. Tukey’s test. a, Doses 10 μg, 30 μg and 100 μg versus 0 μg, p < 0.002. b, SWCNT versus MWCNT, p = 0.036. Two-way ANOVA, PM, p = 0.137. (B) A549 cells. Three-way ANOVA, Dose, p = 0.087; Mod, p = 0.096. Two-way ANOVA, Dose, p = 0.079.

Table 4. Potencies of carbon nanotubes.

<table>
<thead>
<tr>
<th>CELL</th>
<th>PM</th>
<th>β</th>
<th>LDH</th>
<th>ATP</th>
<th>β_{LDH/ATP}</th>
<th>CTB</th>
<th>β_{CTB/BrdU}</th>
</tr>
</thead>
<tbody>
<tr>
<td>J774</td>
<td>SW-P</td>
<td>-0.054</td>
<td>-0.119</td>
<td>-0.087</td>
<td>-0.017</td>
<td>-0.104</td>
<td>-0.061</td>
</tr>
<tr>
<td></td>
<td>SW-O</td>
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J774/A549 group represents the mean potency values for the two cell lines. The grey-shaded lines indicate the highest potencies in each group of cell lines.
attributable to surface functionalities of the CNTs, while high cytotoxic potency for the pristine CNT variants in the LDH and ATP assays ($\beta_{LDH/ATP}$) may be related to metal poisoning of the cells (Shanker, 2008), although additional experiments are required to identify the exact mechanisms of action. Independent of the effect of modification (pristine versus oxidized), our results suggest a possible effect of the type of CNT as well (single-walled versus multi-walled). Single-walled CNTs were slightly more potent than multi-walled CNTs in the BrdU assay (Figure 8A, CNT main effect, $p = 0.036$) and ATP assay (Figure 7A, CNT main effect, $p = 0.064$) of J774 cells, while multi-walled CNTs were more potent than single-walled CNTs in the CTB assay (Figure 6A, CNT main effect, $p = 0.054$).

The fact that pristine CNTs with relatively higher metal contents compared to their oxidized counterparts affect cellular ATP and LDH levels but not CTB and BrdU profiles can be attributed to their impact on cellular energy metabolism, which in turn can affect the cell membrane integrity by decreasing Na+/K+ pump activity and cell calcium homeostasis that is implicated in membrane damage. Moreover, severe ATP depletion is associated with lipid defects, and extensive membrane damage.

However, the levels of cellular ATP that we encounter in this study, even at the highest dose of pristine CNT, are five-fold above the levels where one would start seeing extensive damage (Venkatachalam et al., 1988). Therefore, it appears that at the doses used in this study we are likely observing perturbations of these pathways. On the other hand, oxidized CNTs with relatively increased surface polar functionalities affected oxido-reductive metabolic functions and cellular proliferation. In essence, these observations demonstrate that different types of CNTs affect different biological pathways.

Correlation analyses between CNTs’ physico-chemical properties and average relative potency values estimated based on the two distinct groups of cytotoxicity endpoints for both cell types revealed interesting relationships (Figure 9). For instance, total metal content and surface area were positively correlated with the relative potency values estimated using cellular ATP and LDH levels. Nevertheless, surface polarity was positively correlated with the relative potency values estimated based on CTB and BrdU levels. These observations suggest that the cellular ATP and membrane integrity can be affected by surface area and metal content of the CNTs. Meanwhile, our findings also suggest

![Figure 9](image-url)

Figure 9. Correlation of cytotoxic potency of CNT variants with physico–chemical characteristics. Average cytotoxic potency for the CTB and BrdU assays (A, C, E) and for the cellular contents of ATP and LDH (B, D, F) in both J774 and A549 cell lines expressed as a function of % metal content (A, B), surface area (C, D) and polarity (E, F) of carbon nanotube variants.
that surface polar groups can potentially participate in redox-cycling processes engaging in CTB reduction and oxidative stress-related reactions which can cause DNA damage that can lead to reduced cell proliferation (Neofytou et al., 2012) and thus decreased BrdU incorporation. Future in-depth proteomic and metabolomic analyses can clarify these toxicity pathways and assist in reliably assessing their associations with physico-chemical properties of CNTs.

Our results clearly show that physico-chemical properties of CNTs, such as surface area, catalytic metal content, surface polarity (hydrophilic and hydrophobic properties), have the potential to affect various cellular processes in macrophages and epithelial cells (Figure 9). However, a reductionist approach of correlating individual CNT properties with the apparent cytotoxicity in a selected assay can be misleading.

Conclusions

CNTs are cytotoxic to human epithelial A549 and murine macrophage J774 cells in vitro. Our results indicate that the cytotoxic potency of CNTs is assay-dependent and cell-dependent, revealing that the CNT variants affect different cellular processes to different extents. The findings demonstrate the importance of using multiple cellular cytotoxicity assays and cell types to define toxicity, along with proper physico-chemical characterization of nanomaterials. Although cytotoxic potency and the cellular processes impacted are influenced by the physico-chemical properties of CNT variants, it remains difficult to attribute potency to independent factors such as surface area, pore volume, total metal content and surface polarity. Cytotoxicity data provide a contrasting pattern of functional alterations on which future high-content and detailed pathway analyses can be anchored to elucidate underlying molecular mechanisms of action.

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Declaration of interest

This work was supported by Health Canada (4320105 and Chemical Management Plan). The authors declare no competing interests. Although the content of this communication reflects the work and views of the authors, Health Canada managers review, comment and approve manuscripts before publication.

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