BIOAVAILABILITY OF NICKEL IN SINGLE-WALL CARBON NANOTUBES

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INTRODUCTIONS

The world-wide nanotechnology movement is devoting a rapidly growing literature on biological interactions of carbon nanotubes (CNTs), however the conflicting data have emerged about the biocompatibility and toxicity of carbon nanotubes, and there is no consensus on the overall risk to human health (Hurt et al., 2006; Smart et al., 2006). To resolve this issue, further study is needed, with particular emphasis on understanding the material features of carbon nanotubes, such as size, shape, surface chemistry, and metals content, and we hypothesize that some of the conflicting toxicity data are due to real sample-to-sample variation in these material properties. It is also unclear whether carbon nanotube catalyst residues can trigger these toxicity mechanisms due to apparent encapsulation of the nickel within carbon shells.

Nickel, the majority component in Ni-Y catalysts commonly used in the commercial synthesis of single-wall nanotubes (SWNTs) (Journet et al. 1997), is an established human carcinogen that induces gene silencing and hypoxia signaling through mechanisms involving intracellular nickel cation. The early toxicology literature includes nickle effects in SWNT by Lam et al, nickel nanoparticles and mostly nickel compounds such as nickel subsulfide (Lam et al. 2004; Zhang et al. 1998; Costa et al. 2005). Models of nickel induced respiratory tumors suggests that cellular bioavailability of nickel (the delivery of Ni (II) ions to the nucleus of target epithelial cells) is the major determinant for the carcinogenicity of nickel. A key material science question, therefore, is whether sufficient Ni (II) ion can be released from CNT samples. Moreover, the ability of various nickel compounds to be taken up by cells directly influences intracellular nickel levels, and their carcinogenic activity is proportional to cellular uptake(Oller et al., 1997; Costa et al., 1980). Soluble nickel ions can be transported directly, and insoluble or relatively insoluble nickel compounds can be taken up as particles by endocytosis or phagocytosis. Phagocytosis/endocytosis of poorly soluble nickel compounds such as Ni_3S_2 and NiO may play an important role in the pulmonary toxicity and carcinogenicity of nickel. The "nickel-ion hypothesis" states that the nickel toxicity, mutagenesis and carcinogenesis mainly depend on the intracellular nickel(II) ion concentration, independent of the original nickel compounds to which an organism is exposed (Snow et al., 1998; Fletcher et al. 1994) .

Overall, the existing literature is sufficient to raise the issue of nickel effects in the biological response to CNTs, but there is major uncertainty due to the lack of information about the *bioavailability* of CNT-associated nickel. Nickel mobilized from carbon nanotubes extracellularly may enter cells through the divalent metal transporter (DMT1) and other possible ion channels (Gunshin et al., 1997). A specific concern for CNTs is the possibility that phagocytosis / endocytosis of the nanotubes or their aggregates could provide a route of entry into cells for the Ni nanoparticles associated with those CNTs (Kam et al., 2004). Following uptake, the low pH environment within lysosomes may cause Ni(II) release in significant quantities through an acid corrosion mechanism. The biologically active species is reported to be Ni^{2+}, which disrupts the hydroxylation of transcription

factor HIF1-α or induces gene silencing by binding to heterochromatin (Costa et al., 2005; Oller et al., 2002). The most significant unknown is the nickel release behavior of CNTs (bioavailability), which should be characterized under both extra- and intracellular conditions.

In this paper, we designed appropriate cellular biological assays for Ni bioavailability based on the anticipated pathways and mechanisms involved in the cytotoxicity of Ni-containing carbon nanotubes and show the toxicologically significant amounts of nickel can be mobilized into model physiological fluids from a range of commercial nanotube samples. We quantify the extent of Ni^{2+} mobilization as a function of pH, dissolved oxygen, dissolved salts, and post-processing involving sonication, oxidation, and mechanical grinding. We also report on the intracellular uptake of CNT-nickel by human lung epithelial cells to quantify its bioavailability relative to soluble $NiCl_2$ as a known reference nickel source. These results suggest practical methods for managing nickel residues to minimize nanotube health risks.

EXPERIMENTAL SECTION

Materials and nickel mobilization assay

As-produced and purified single-wall carbon nanotube samples were acquired from four different vendors, identified here only by code (A-D), mostly all of the CNT samples contains Ni and Y(see table 1), whose contents were determined by inductively coupled plasma atomic emission spectroscopy(ICP).

Materials characterization was carried out using a JEOL JEM-2010 TEM at 200 kV and a Bruker AXS D8 Advance X-ray Diffractometer. Elemental analyses of single-wall nanotube samples were performed by ICP-MS analysis at Huffman Laboratories, Inc (Golden, CO).

Table 1 Residual catalytic metal contents of a range of commercial SWNTs samples

Vendor	SWNT product description	Catalyst (wt%)
A	As-produced (AP)	Ni 19.4% Y 5.49%
	Purified	Ni 14.3% Y 2.09%
B	As-produced	Ni 3.15% Co 9.21%
C	As-produced	Ni 22.8% Y 4.79%
D	As-produced	Ni 24.1% Y 4.17%
	High Purity	Co 3.30% Mo 1.27%

Standard nickel mobilization assays were carried out by sonicating single-wall nanotube suspensions (1.0 mg/ml) in a bath sonicator for 2 h after which the suspensions were transferred to 5000 NMWL Amico centrifugal filter devices (Millipore, MA) and subjected to 1~2 h of centrifugation at 4000 rpm to remove the carbon nanotubes and any free Ni nanopaticles. In this way, a clear nickel(II)-containing solution could be obtained free of suspended solids. These experiments were carried out in a variety of different aqueous solutions, including de-ionized (DI) water, deoxygenated DI water, acidic buffer at a typical lysosomal pH (5.5 in acetate buffer), deoxygenated acidic buffer, phosphate-buffered saline (PBS) and cell culture media (RPMI 1640). The experiments in media used centrifugation at 4^{o} C, but otherwise the experiments were carried out at room temperature.

Cell culture and measurement of intracellular nickel concentrations

Human lung epithelial H460 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI medium (Invitrogen, CA) supplemented with 10% Fetal Bovine Serum (Gemini, CA) and 1% Penicillin-Streptomycin (Invitrogen, CA) in a 37^{o} C cell culture incubator containing 5% CO_2. Cells were exposed to RPMI1640 extracts of single-wall nanotubes (SWNTs), activated carbon, or acid washed SWNT for 24 hours. Control sets of cells were treated in parallel with the same batch of RPMI1640 medium doped with 0, 100 µM or 200 µM $NiCl_2$ (Sigma, MO). At the end of 24 hours, cells were rinsed with cold PBS and collected with trypsinization. Cells were washed twice with cold PBS and collected by centrifugation at 1100 g at 4^{o} C. Cell pellets were resuspended in 100 µl of 5 % nitric acid, heated at 50^{o} C for 60 minutes and then placed on ice for 30 min before centrifugation at 10,000 g at 4^{o}C for 10 min. Metal-containing supernatants were collected and diluted 2.5 times with water to bring the nitric acid concentration to 2 % and stored at 4^{o} C for analysis with graphite furnace atomic absorption spectroscopy. Pellets were washed twice with 200 µl of cold 5 % nitric acid and centrifuged for 5 min at 10,000 g at 4^{o} C, and dissolved in 200 µl of 0.5 M NaOH. After incubation of pellets with NaOH for 30 min at 37^{o} C, protein concentrations of the samples were determined using an SDS-compatible protein determination kit from Bio-Rad. Final results were expressed as total amount of Ni divided by total amount of protein in each individual tube (pg Ni/mg protein).

RESULTS and DISCUSSIONS

Figure 1 shows the HRTEM images of six commercial SWNT samples chosen for this study as well as information on typical metal morphology. The metal is primarily in the form of metallic nanoparticles coated with carbon shells (2 – 10 nm thick), which range from amorphous (panel a) to

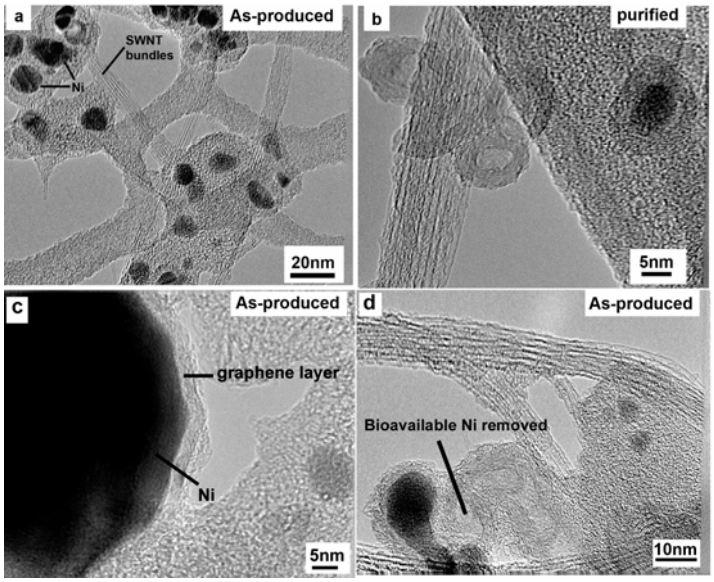

Figure 1. Typical morphologies for Ni-containing commercial SWNTs. a, c: As-produced from vendor C; Fig. 1d and 1b are as-produced and purified from vendor A.

graphenic with well-developed concentric order (panel b). The extent to which these carbon shells provide protection from fluid access is unclear due to ultrafine porosity in the amorphous carbon and/or to defects in the ordered carbon that are not readily seen in the two-dimensional projection of TEM. Panel b illustrates the limitations of TEM – these hollow shells appear to be intact and protective, but logically cannot be, since the original internal metal particle has apparently been removed during vendor purification by a process that requires fluid access by small-molecule mineral acids. Panel c and d are as-produced SWNT after 2hrs incubation at 1.0 mg/ml in acetic buffer of pH5.5 at room temperature. Panel c shows thin but perfectly crystalline graphene layers which can protect encapsulated metal catalyst very well, however, empty carbon shells found in panel d suggest that small molecules can diffuse through ultrafine defects in some carbon shells in this assay and remove the bioavailable nickel.

Figure 2 shows the nickel mobilized into simple aqueous phases upon 2 hr exposure at a dose of 1.0 mg-CNT/ml. Significant quantities of Ni are released from each of the samples except the Co/Mo-containing CNT sample used as a negative control. Nickel mobilization is greatly enhanced under mildly acidic conditions (at typical lysosomal pH of 5.5) compared to extracellular pH (near 7) and is weakly enhanced by the presence of dissolved oxygen, suggesting an acid corrosion mechanism.

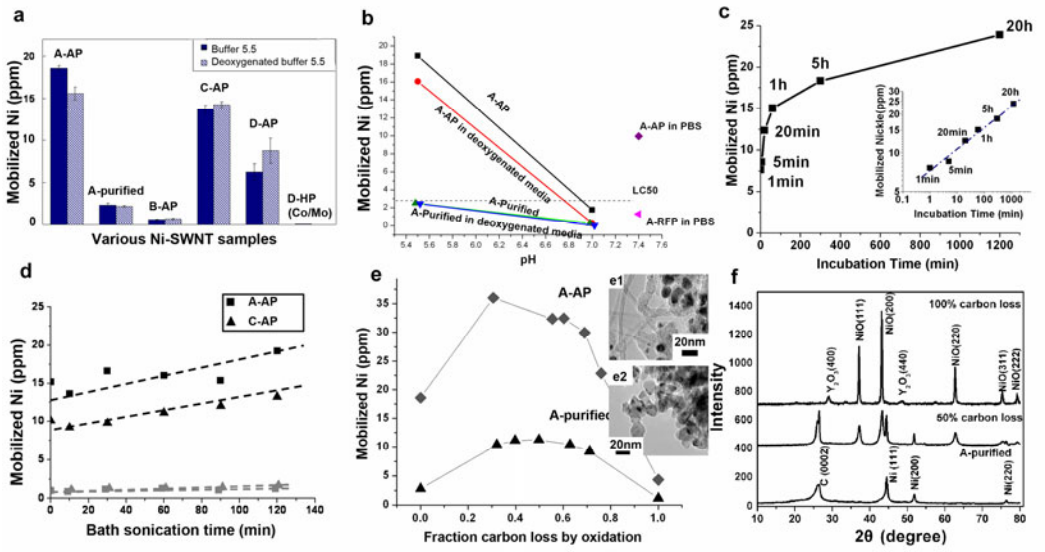

Figure 2. Mobilization of nickel from SWNTs into simple fluid media by ICP analysis. Fig.2a. Nickel mobilization into lysosomal pH of 5.5 acetic buffer. Fig.2b. Effect of pH and comparison with previously reported cytotoxicity LD50 values for Balb/3T3 clone A31 fibroblast cells from Taira et al. (2001). Fig.2c. Time release study at pH 5.5 for the as-produced SWNT sample from vendor A. Fig.2d. Detailed examination of mobilization dynamics during bath sonication at pH 5.5 (upper two curves) and pH 7 (lower two curves). Fig.2e Effect of air oxidation to simulate oxidative purification processes for removal of amorphous carbon (inset e1 untreated A-AP and e2 100% oxidized A-AP). Fig2f. XRD patterns for A-purified sample before and after air oxidation.

The sample-to-sample variation is striking, ranging (at pH 5.5) from 18 ppm to less than 1 ppm, which represents about 2-9% of the total sample nickel similar (at neutral pH) or much higher (at low pH)

than those previously reported to cause cytotoxicity (LD50) in murine fibroblasts, and should be regarded as toxicologically significant. Figure 2c shows that CNT-nickel release is initially rapid and slows gradually but does not reach an asymptote within the 20 hr experiment. The linear log-log behavior (inset) indicates a power-law release, $N = Kt^n$ with K = 7.3 and n = 0.17 for t in minutes and N in ppm. The release profile is important, since the early release will occur extracellularly, while late release can occur intracellularly in acidified endosomes enhancing access to the nucleus and increasing toxicity.

Figure 2d-f shows the effects of sonication as well as other processing methods on Ni mobilization to test this hypothesis. Bath and probe sonication, both widely used to improve CNT dispersion in fluids, are seen to increase nickel mobilization after both 10 min and 2 hr. We wish to emphasize that sonication is *not required* for nickel mobilization – even with no sonication whatsoever, significant Ni mobilization occurs, implying that some portion of the Ni is fluid accessible even without post-processing shell damage. Air oxidation is seen to either enhance or suppress mobilization depending on the sample and extent of carbon consumption. It appears that oxidation can expose encapsulated metal (enhancement) but can also oxidize the metal to the more thermodynamically stable NiO with correspondingly lower free metal release (see in inset Fig2e HRTEM and Fig2f XRD). Mortar and pestle grinding for 10 - 15 min had no significant effect on Ni mobilization for either the AP or purified samples from vendor A (data not shown).

We studied the interaction of the interaction of H460 human lung epithelial cells with SWNT from Vendor A for 48hrs. As shown in Figure 3, single SWNT or small aggregates are actively taken up by receptor-mediated endocytosis or phagocytosis, which may subsequently increase toxicity by releasing metallic catalyst residues to organelles or nuclear DNA.

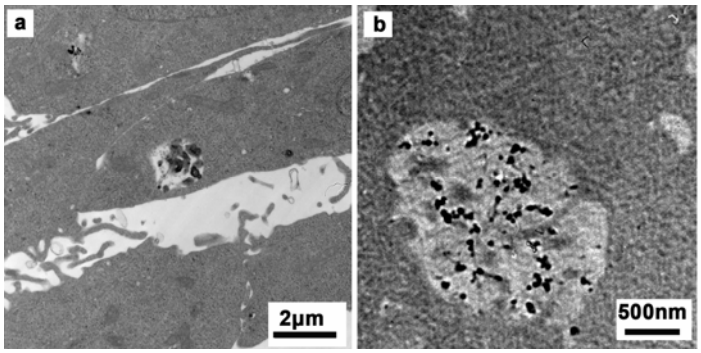

Figure 3. TEM thin sections showing SWNT internalization into H460 cells.

Figure 4 illustrates the behavior of Ni when SWNTs are incubated in RPMI 1640 cell culture media. Nickel mobilization in this complex environment (RPMI contains over 40 components) is significantly enhanced from mobilization in de-ionized water (see in Fig.4a). Figure 4b shows that Ni mobilized from CNTs directly into RPMI medium, followed by centrifugal separation of the CNTs, is bioavailable to H460 human lung epithelial cells, resulting in intracellular/extracellular Ni ratios that are higher than those for the soluble reference salt, $NiCl_2$.

We hypothesize that enhanced bioavailability of CNT-nickel relative to soluble $NiCl_2$ is due to extracellular interactions between SWNT and medium that reduce the ligand concentrations which would limit intracellular uptake of the Ni(II) cation. To test this hypothesis we pre-incubated the

media with SWNTs that had been previously washed with HCl in order to remove the bioavailable fraction of the nickel residue, and then removed the nanotubes by ultrafiltration. (Note: use of these treated SWNTs introduced only an insignificant amount of Ni (0.1 ppm) to the medium so the main effect of this pretreatment was the interaction of SWNT carbon with the medium components). The pretreated media were then doped with $NiCl_2$ and the experiment in Fig. 4b repeated, yielding the results in Fig. 4c. This pretreatment increased the uptake of nickel from $NiCl_2$, demonstrating that SWNT/medium interactions are responsible for at least part of the unusually high bioavailability of SWNT-derived nickel. Repeating this experiment with commercial activated carbon particles (FLUEPAC^{TM}-B from Calgon Carbon surface area 1115 m^2/g) produces a similar effect, suggesting that the SWNT-medium interactions are not unique to SWNTs, but are rather related to the presence of high-surface-area carbon.

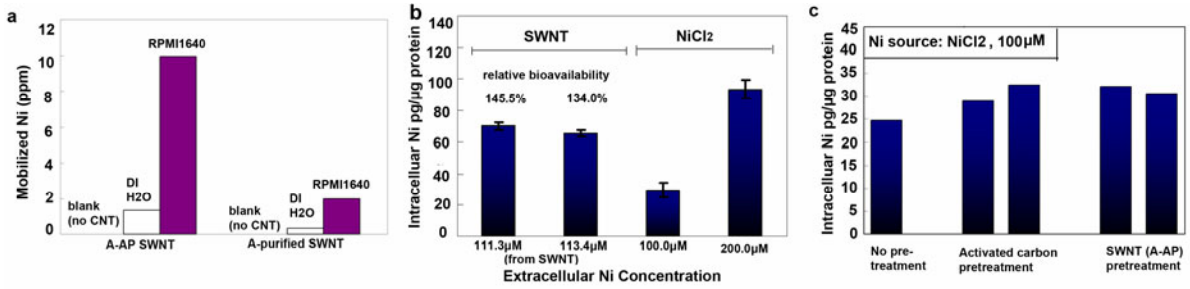

Figure 4. SWNT nickel release into complex physiological fluids and its subsequent uptake into human lung epithelial cells, H460. (a) Ni mobilization is enhanced in cell culture media relative to simple fluids at neutral pH, all at 1.0 mg-SWNT/ml. (b) Total intracellular Ni after 24 hr exposure of H460 cells to media previously incubated with SWNTs; also after 24 hr exposure to media containing $NiCl_2$ salts as reference. (c) Pre-exposure of the medium to high surface area carbon materials at 1.0 mg/ml is seen to enhance the uptake of $NiCl_2$. Multiple bars represent duplicate tests.

Finally, these results suggest that nanotube health risks may be minimized by targeting the bioavailable portion of the residual nickel for removal (rather than targeting the total nickel which is a

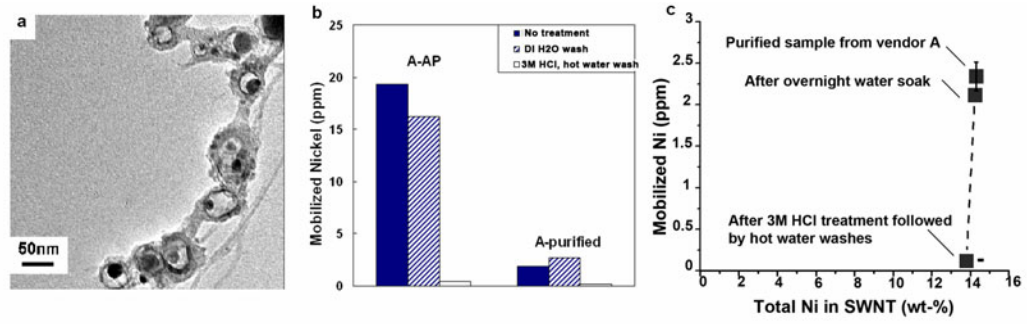

Figure 5. Reduction in Ni bioavailabilty through post-processing. A. TEM image of as-produced SWNT from vendor A after 48hr HCl wash; b. Nickel mobilization of AP and Purified SWNT from vendor A in PSF for different post-treatments; c. The residual free nickel remaining after the original vendor purification process can be greatly reduced by treatment with 3 M HCl and hot water washing. Values are mean $\pm$ s.e. (shown on or adjacent to point) in pH 5.5 buffer.

challenge to remove completely). Treating the sample with 3 M HCl for 48 hrs followed by hot water washes (to avoid surface salt deposition) reduces Ni mobilization in phagolysosomal stimulant fluids (PSF) (Stefaniak , et al. 2005) dramatically (see in Figure 5b).

Another example is demonstrated in Fig. 5c using as a starting point the vendor purified sample, which surprisingly still exhibits significant Ni mobilization. Treating that sample with 3 M HCl for 20 hrs followed by hot water washes reduces Ni mobilization from 2.3 ppm to about 0.12 ppm. This procedure avoids air oxidation and oxidizing acids, which attack the carbon shells and provide access to previously encapsulated metal, and thus the bioavailable portion is selectively targeted and the bulk metal is largely left intact and biologically unavailable (see dashed line in Fig. 5c). Although these simple procedures greatly reduce bioavailable metal, they do not typically eliminate it, and sub-ppm Ni release is often observed after treatment. The partial empty carbon shells in as-produced SWNT in Fig. 5a suggest that the persistence of some bioavailable nickel may be due to slow dissolution within some difficult-to-access carbon shells, which is in consistent with the extended dynamic nickel release curve in Fig2c.

CONCLUSIONS

Overall, we have shown that nickel in a variety of SWNT samples, both as-produced and "purified", is bioavailable at toxicologically significant concentrations and that bioavailability can be enhanced or suppressed by various nanotube post-processing scenarios. There is potential to reduce SWNT health risks by more intelligent management of the imbedded nickel residues, including targeted removal of the bioavailable fraction in a manner that does not damage the carbon shells and expose fresh metal.

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