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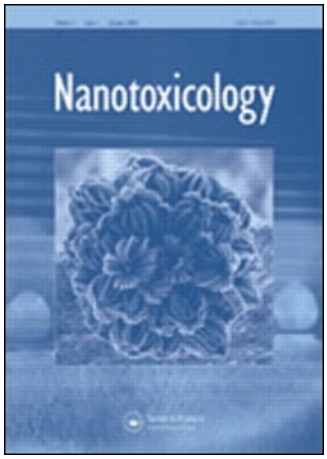

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Enhanced **in vitro** and **in vivo** toxicity of poly-dispersed acid-functionalized single-wall carbon nanotubes

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Enhanced *in vitro* and *in vivo* toxicity of poly-dispersed acid-functionalized single-wall carbon nanotubes

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Abstract

Many potential applications in nanotechnology envisage the use of better-dispersed and functionalized preparations of carbon nanotubes. Single-walled carbon nanotubes (SWCNTs) were treated with 1:1 mixtures of concentrated nitric and sulfuric acids for 3 min in a microwave oven under 20 psi pressure followed by extensive dialysis to remove the acids. This treatment resulted in acid functionalized SWCNTs (AF-SWCNTs) that had high negative charge (Zeta potential −40 to −60 mV) and were well dispersed (98% of the particles <150 nm) in aqueous suspensions. *In vitro* and *in vivo* toxic effects of SWCNTs and AF-SWCNTs were compared. AF-SWCNTs exerted a strong cytotoxic effect on LA4 mouse lung epithelial cells in culture that could be blocked by prior treatment of the nanotubes with poly L-lysine which neutralized the electric charge and promoted re-agglomeration. AF-SWCNT, but not the unmodified SWCNT preparations, strongly inhibited cell cycling of LA4 cells. Both SWCNTs and AF-SWCNTS were however equally effective in inducing apoptotic responses in LA4 cells as examined using an Annexin V binding assay. Oro-pharyngeal aspiration of AF-SWCNT preparation induced a strong acute inflammatory response in the lungs of CD1 mice, compared to control SWCNTs which caused only a marginal effect. Taken together the results indicate that unlike pristine SWCNTs, acid-functionalized SWCNT preparations exert strong toxic effects *in vitro* and *in vivo* and these effects can be reversed by neutralizing their surface charge.

Keywords: *Nanoparticles, nanotubes, particle toxicology*

Introduction

Carbon nanotubes are finding wide applications in aerospace, automobile, electronics and solar cell industries because of their unique structure and properties. Additionally, carbon nanotubes are being investigated extensively in biomedical and pharmaceutical fields for tissue engineering, drug delivery systems and as biosensors (Balasubramanian & Burghard 2006; Lacerda et al. 2006; Shiba 2006; Harrison & Atala 2007). Structurally, single-walled carbon nanotubes (SWCNTs) represent rolled up graphite-like sheets of sp2 hybridized carbon atoms, and like graphite, have great affinity for adhering to each other resulting in large, difficult to disperse agglomerates (Fu & Sun 2003).

Improved dispersion of nanotubes to form stable suspensions in aqueous media would allow for a wider range of chemical and physical manipulations and would facilitate even wider applications for SWCNTs. Considerable efforts have therefore been made to render nanotubes stably dispersed or solubilized in water and organic solvents (Chen et al. 1998; Georgakilas et al. 2002; Peng et al. 2003; Schlecht et al. 2003; Qin et al. 2004, Fernando et al. 2005, Zhao et al. 2005). While most of these methods only marginally improve the solubility of nanotubes, a procedure recently reported by Wang et al. (2006) yielded SWCNTs with much greater dispersion. The technique involves reacting SWCNTs with mixtures of sulfuric and nitric acids in a high pressure microwave oven, and results in extensive derivatization of the carbon backbone with carboxylic acid and sulfonate groups (Wang et al. 2006). While the acid functionalized SWCNTs (AF-SWCNTs) retain their structure, they are efficiently de-bundled and disperse easily in aqueous solution (Wang et al. 2006).

The increasing potential applications of pristine as well as dispersed carbon nanotubes provide strong

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impetus to investigate potential toxic effects of these materials. Several reports on the toxicity of carbon nanotubes already exist in literature (reviewed in Lam et al. 2006). Huczko and Lange (2001) found no effect of intra-tracheal instillation of CNTs on pulmonary functions of guinea pigs. Lam et al. (2004) tested a variety of SWCNT samples with varying amounts of metal impurities and concluded that all SWCNT preparations induced dose-dependent lung granulomas in mice. Warheit et al. (2004) reported a mild and transient pulmonary inflammatory response in rats instilled intra-tracheally with SWCNTs, with subsequent development of multifocal granulomas in the lungs after 1 month. In a mouse instillation study using highly purified SWCNTs, Shvedova et al. (2005) found granulomas, lung fibrosis and a significant elevation in markers of toxicity in broncho-alveolar lavage (BAL) fluid, and concluded that SWCNTs exerted greater toxicity on a mass basis than crystalline silica. In contrast, no adverse effects were observed in rabbits injected intravenously with low levels of surfactant dispersed SWCNTs (Cherukuri et al. 2006), and no significant toxic effects were seen following incubation of SWCNTs with cultured A549 human lung epithelial cells (Davoren et al. 2007; Pulskamp et al. 2007).

An important and unregulated variable in these studies has been the dispersibility of SWCNT preparation. In the present study, we compared the toxic effects of pristine and AF-SWCNTs, and examined their effects on viability and cell cycling of LA4 mouse lung epithelial cells. We then extended the studies to *in vivo* pulmonary toxicity measurements in mice after oro-pharyngeal aspiration of the same material. The results confirm that acid functionalization greatly increases dispersibility and surface charge of SWCNTs, and show that these changes are associated with enhanced toxicity in the *in vitro* and *in vivo* assay systems.

Materials and methods

Cells and reagents

The LA4 murine lung epithelial cell line was obtained from American Type Cell Culture and maintained in RPMI 1640 culture medium supplemented with glutamine (2 mM), HEPES buffer pH 7.2 (25 mM), gentamycin (20 μg/ml), and fetal bovine serum (10% V/V). Hamster anti-mouse FAS monoclonal antibody, propidium iodide/RNase staining buffer and 7AAD/Annexin V-PE kit for studying apoptosis were obtained from BD Pharmingen, San Jose, CA, USA. Single-walled carbon nanotubes (SWCNTs) were procured from Sigma (Catalog no. 636797, amorphous carbon <3%). While representative data has been shown using the SWNCT preparation from Sigma and its acid functionalized derivative, similar results were also obtained for SWCNT preparations procured from other two sources [Carbon Nanotubes Inc. Houston, TX, USA (purified HIPCO SWCNTs <15% ash) and Cheap Tubes Inc. Brattleboro, VT (purity >90%)] and their derivatives.

Animals

CD1 female mice were obtained from Charles River and used between 12 and 16 weeks of age (average body weight 31 g $\pm$ 2.6). All mouse experiments were conducted after due approval of the Institutional Animal Care and Use Committee.

Acid functionalization of SWCNTs

Acid functionalization of SWCNTs was carried out using the procedure described by Wang et al. (2006). In brief, 20 mg SWCNT samples were suspended in 20 ml of 1:1 HNO_3:H_2SO_4 (both Optima grade, Fisher Scientific, Pittsburgh, PA, USA) in 100 ml high-pressure vessels and placed in a microwave digester (Model UDV-10 vessels, MDS-2100 digester, both from CEM Corporation, Matthews, NC, USA). Microwave power was applied at 50% of 900W total and pressure controlled at 20 $\pm$ 2psi for 3 min resulting in an internal temperature of 138–150°C. Suspensions were cooled, diluted five times with H_2O and dialyzed four times against 5 l of H_2O over a two-day period. Dialyzed suspensions were dried by lyophilization, weighed and resuspended at the desired concentration in water or saline. At this stage the suspensions were black, well dispersed and had a neutral pH.

Determination of particle size and zeta potential distributions

A Zetasizer Nano (Malvern instruments, Malvern, UK) was used for measuring particle size and zeta potential (ZP) distributions based upon the dynamic laser scattering properties of nanotubes in aqueous suspensions. AF-SWCNTs were suspended in water or saline at a concentration of 20 μg/ml, and transferred to the appropriate measurement cuvette supplied with the instrument. Only those results that passed the instrument's built-in quality control criteria were accepted (www.malvern.com).

Cell cycle and apoptosis measurements

LA4 cells were seeded at a concentration of 2×10^4 cells/ml in 6-well culture plates. When cells had

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reached half confluency, test nanotubes preparations were added at the desired concentration and the cultures were continued for 24 h. LA4 cell monolayers were washed with ice cold PBS and cells harvested by trypsinization, washed once with complete culture medium and then with PBS containing 1% BSA. The cell pellets were resuspended in 0.2% Igepal CA-630 (Sigma) in PBS and vortexed for 1 min. Cells were washed once more with 1 ml PBS containing 1% BSA and suspended in 0.5 ml propidium iodide/RNase solution (Sigma chemicals Inc). After 30 min incubation in the dark at room temperature, cells were analyzed on a BD FACScaliber flowcytometer. For apoptosis assessment, cells were double stained with 7AAD (a dye that enters dead cells and binds to DNA) and Annexin V PE, which binds phosphatidylserine expressed on the outer membrane of apoptotic cells (www.bdbiosciences.com). Staining and flow cytometric assessment were performed according to the manufacturer's instructions.

Oropharyngeal aspiration and BAL analysis

SWCNTs or AF-SWCNTs were suspended in normal saline at a concentration of 800 μg/ml, and 50 μl of this suspension (40 μg per mouse; average 1.25 mg/kg) was injected into the oropharynx and aspirated described previously, (Gilmour et al. 2004). Briefly, mice were anesthetized in a small plexiglass box using vaporized isoflurane (Webster Veterinary, Sterling, MA, USA) and suspended vertically by their front incisors on a small wire attached to an inclined stainless steel support apparatus. The tongue was extended with forceps and 50 μl of saline (Sigma, St Louis, MO, USA) alone, or the SWCNT or AF-SWCNTs mixture was pipetted into the oro-pharynx. The nose of the mouse was then covered causing the liquid bolus to be aspirated into the lungs.

At 18 and 72 h post dosing, four mice from each treatment group were euthanized with sodium pentobarbital. The trachea was then exposed, cannulated, and secured with suture thread. The thorax was opened and the left lung lobe clamped with a micro-hemostat. The right lung lobes were lavaged three times with a single volume (35 ml/kg body weight) of warmed Hanks balanced salt solution (HBSS). The resulting lavage fluid was centrifuged (717 ***g***, 10 min, 4°C) and an aliquot stored at either 4°C for biochemical measurement or −70°C for cytokine assays. The pelleted cells were resuspended in 1ml of HBSS and 0.5 ml of the cell suspension was used for total cell counts with a coulter counter. The remaining sample was centrifuged on to duplicate slides using a Cytospin (Shandon, Pittsburgh, PA, USA) and stained with Diff Quik solution (American Scientific, McGraw Park, IL, USA) prior to differential cell count under light microscopy where at least 200 cells were counted per slide.

Cytokine measurements

Pro-inflammatory cytokines MIP-2, tumor necrosis factor–alpha (TNF-α) and interleukin–6 (IL-6) concentrations in BAL were measured by ELISA using mouse Quantikine kits purchased from Biosource (Camarillo, CA, USA). The assays were carried out as per the manufacturer's instructions.

BAL Biochemistry

Lactate dehydrogenase (LDH) and total protein were measured in a Konelab centrifugal spectrophotometer (Thermo, Finland). Activity for LDH was determined using commercially available kits from Sigma, (MO). Total protein concentrations were determined with the Comassie Plus protein assay (Pierce Rockford IL, USA).

Statistical analysis

Non-paired Student's t-test was used to test for significance of differences between different sets of data.

Results

Effect of acid-functionalization on the physico-chemical properties of SWCNT

SWCNT preparations were highly agglomerated and even after prolonged sonication did not disperse in normal saline or culture medium with 10% FCS. Acid-functionalization of SWCNTs resulted in highly dispersed and stable dark-colored suspensions in aqueous media. Figure 1 (top panel, extreme right well) shows that the highly agglomerated and insoluble SWCNTs when suspended in aqueous suspension, settled to the bottom. In contrast, AF-SWCNTs were well dispersed in aqueous suspensions at the various concentrations (Figure 1 top panel). Wang et al. (2006) have shown that the process of acid functionalization did not alter the structural integrity of the carbon nanotubes. Our results of scanning electron microscopy confirm these findings. Figure 1 (lower panel A) shows the scanning electron microscopic image of control SWCNTs that were highly agglomerated with some nanotube bundles jutting out of the aggregated mass of SWCNTs. Panel B (Figure 1) depicts an SEM image of a lyophilized AF-SWCNT preparation. More discrete bundles of AF-SWCNTs could

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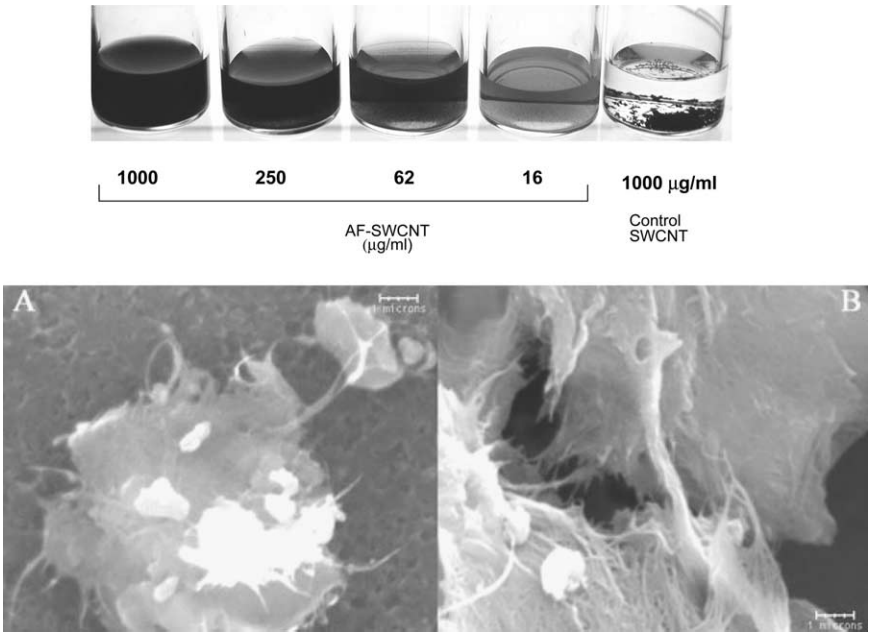

Figure 1. Dispersibility in saline and scanning electron micrographs of control and acid functionalized SWCNTs. SWNCTs and AF-SWCNT preparations were suspended at indicated concentrations in water, vortexed and allowed to sit for 5 min before the photograph was taken (top panel). Bottom panel A shows the scanning electron micrograph of pristine SWCNTs. Panel B shows SEM picture of lyophilized AF-SWCNT powder.

be observed within the dried aggregates indicating that the basic nanotube structure was retained after acid functionalization.

For further characterization of AF-SWCNT preparations, distributions of surface charge (Zeta potential or ZP) and particle size were examined by dynamic laser scattering. The results indicate that different batches of AF-SWCNTs comprised >95% particles below 150 nm size and had mean zeta potential ranging between −40 and −60 mV. High negative charge on functionalized nanotubes, (a consequence of the attachment of negatively charged carboxyl and sulfonate groups on AF-SWCNTs), was confirmed by these results. Since a stable aqueous suspension is a pre-requisite for determining size and charge distribution, these properties for highly agglomerated and insoluble control SWCNTs could not be measured.

Effect of control and AF-SWCNTs on the recovery of viable LA4 cells in culture

The effects of control and acid functionalized SWCNTs on the recovery of live LA4 lung epithelial cells in culture were examined. For this purpose, equal numbers of LA4 cells were seeded in wells of a 48-well culture plates. After 24 h, control and acid functionalized SWCNT preparations were added to the culture wells at required concentrations and cultures continued for up to four additional days. After two days (Figure 2, panel A) and four days (Figure 2, panel B), culture wells were washed with PBS and adherent LA4 cells were detached by trypsinization and counted after a 1:1 dilution with 1% trypan blue solution in PBS. Viability of cell preparations at this stage were always above 90% because dead/lysed cells would have been washed away prior to harvesting of the live LA4. Recoveries of LA4 cells were significantly lower when either SWCNTs or AF-SWCNTs were present in the culture. The decline in cell recovery was however much greater in AF-SWCNT treated cells. At the lowest concentration of nanotubes (5 μg/ml) recovery of LA4 cells fell approximately 30% and 85% in the presence of SWCNTs and AF-SWCNTs respectively (Figure 2B). The kinetics of effect of AF-SWCNTs on LA4 cell recoveries is depicted in Figure 2C and show that while control LA4 cells grew exponentially for four days, there was no increase in AF-SWCNT treated cells at any time point.

Effect of control and acid functionalized SWCNTs on cell cycle of LA4 cells

The decline in recovery of viable cells following treatment with SWCNTs and AF-SWCNTs could be due to inhibition of cell proliferation or increased cell death, or both. A depressed cell proliferative activity is preceded by reduced DNA synthetic activity. To assess such changes, LA4 cells were cultured with or without SWCNTs and AF-SWCNTs for 24 h and cell nuclei were isolated for

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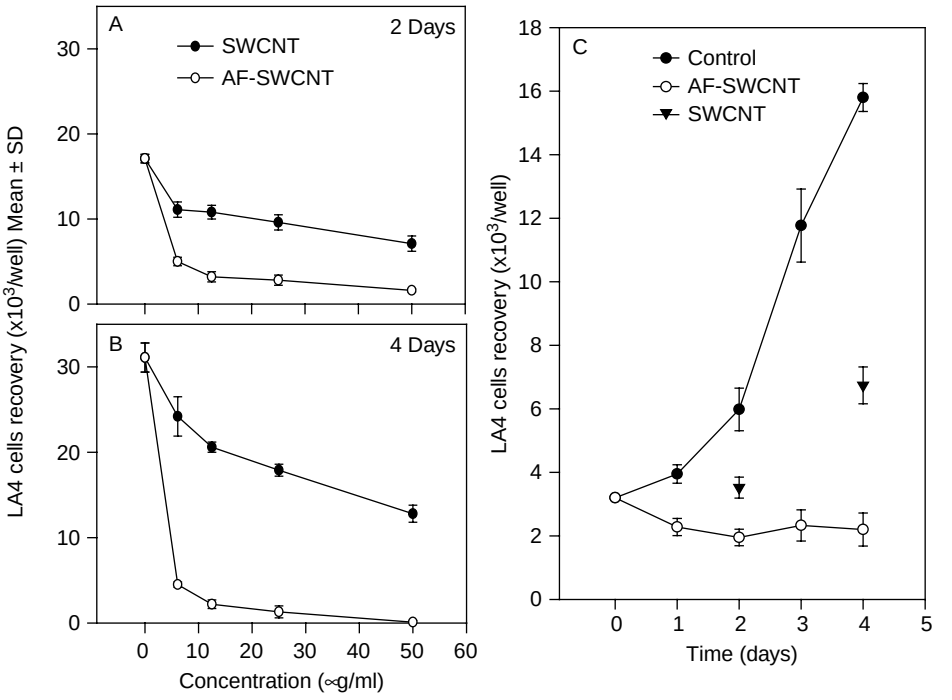
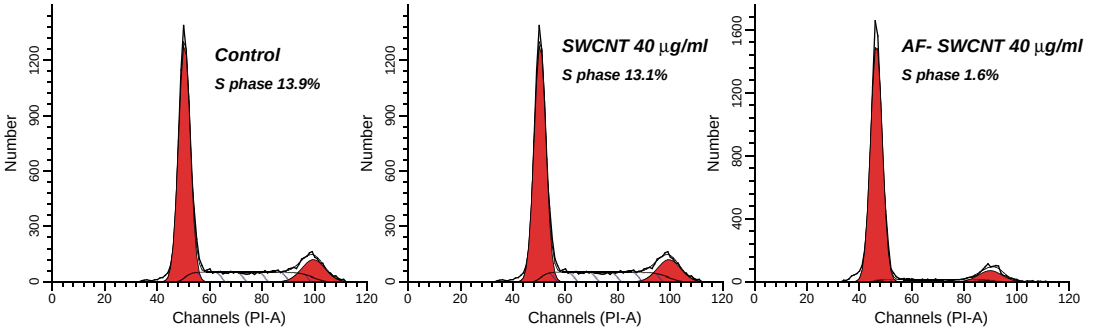


Figure 2. Effect of control and acid functionalized SWCNTs on the recovery of live LA4 lung epithelial cells in culture. LA4 cells were cultured in 48-well culture plates and exposed to different concentrations of control and acid-functionalized SWCNT preparations. After 48 (panel A) and 96 h (panel B), adherent LA4 cells in culture wells were washed, detached by trypsinization and suspended in a 1% trypan blue solution in PBS. Recoveries of viable cell numbers were assessed by cell counting using a hemocytometer. Each point represents mean ± SD of values obtained from 4 replicate assay wells. Inhibition of LA4 cell recoveries was significant at all test doses ($p < 0.0001$–0.05 in different cases). Time kinetics of effect of SWCNTs and AF-SWCNTs (50 μg/ml) is depicted in panel C.

staining with propidium iodide. Representative flow cytometric data in Figure 3 indicate that in control LA4 cell cultures, 13.9% cells were in the S phase of the cell cycle. This value was not affected by adding SWCNT to LA4 cells. However, AF-SWCNT reduced the proportion of S phase cells to only 1.6%. AF-SWCNT treatment also resulted in a significant decrease in cells in G2/M phase and a



Treatment	*Percent cells*		
	G1/G0 phase	*S phase*	*G2/M phase*
None	72.2 ± 2.9	13.9 ± 2.6	13.4 ± 3.3
SWCNT 40 μg/ml	71.2 ± 3.3	13.1 ± 4.8	15.7 ± 1.5
AF-SWCNT 40 μg/ml	90.7 ± 0.6*	1.6 ± 0.5*	7.7 ± 0.2*

Figure 3. Effect of control and acid-functionalized SWCNTs on LA4 cell cycle. LA4 cells were cultured in 25-cm culture flasks with or without 40 μg/ml of SWCNT or AF-SWCNT preparations. After 24 h, LA4 cell monolayers were washed and cells isolated by trypsinization. Cell nuclei were isolated and stained with propidium iodide for flow cytometric analysis as described in Materials and Methods. Data was analyzed by using Modfit software that enumerated proportion of cells in G1/Go phase (left peaks in all histograms), S phase (cross hatched peaks) and G2/M phase (right dark peaks in all histograms). Results of a representative experiment are shown. Values in the table (lower panel) represent mean ± SD of percentages of cells in different phases of cell cycle obtained from four independent estimations. *$p < 0.01$.

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concomitant greater proportion of cells in Go/G1 phase. These results clearly demonstrate that AF-SWCNT treatment significantly inhibited cell proliferation in LA4 epithelial cells.

Induction of apoptosis by SWCNT

The induction of apoptosis by SWCNT and AF-SWCNT in LA4 cells was examined as a possible contributory factor in lower cell recoveries from SWCNT and AF-SWCNT treated cultures. Anti-Fas antibody that is known to induce apoptosis was used as a positive control. Since apoptotic changes precede actual cell death, we examined LA4 cells 24 h after treatment with nanotubes, even though the actual effect on cell numbers was greater at later time points. Control, SWCNT, AF-SWCNT and anti-Fas antibody treated LA4 cells were stained with 7AAD and Annexin V and analyzed by flow cytometry. Annexin V binds with the externalized phosphatidyl serine that is a characteristic of apoptotic cells (van Engeland et al. 1998), and 7AAD stains dead cells by binding with DNA. Representative results in Figure 4 show that 4.9% of control LA4 cells were apoptotic ($7AAD^-$ Annexin V^+), and this percentage increased to 18.3% after treatment with anti-FAS antibody. The mean $\pm$ SD of three replicate experiments for the percentage of apoptotic cells was 4.93 ± 0.84 (control), 11.73 ± 2.80 (SWNCT-treated, $p < 0.01$), 13.87 ± 1.00 (AF-SWCNT-treated, $p < 0.001$) and 16.47 ± 1.63 (Fas antibody, $p < 0.001$). These results suggest that SWCNT as well as AF-SWCNT were equally effective in inducing apoptosis in LA4 epithelial cells. It should be noted that a significant accumulation of $7AAD^+$ dead cells was not detected because dead cells would have been washed away prior to the harvesting of live LA4 cells by trypsinization.

Neutralization of cytotoxic effects of AF-SWCNTs by poly L-lysine

The results so far indicated that the potent inhibitory effect of AF-SWCNTs on recovery of LA4 cells resulted from a combination of cytostatic as well as cytotoxic effects. Highly charged AF-SWCNT preparations were finely dispersed in suspension and these properties could likely promote interactions between the AF-SWCNT particles and the LA4 cells which might account for the potent inhibitory effect of AF-SWCNTs. In order to test whether charge itself was responsible for this effect, we

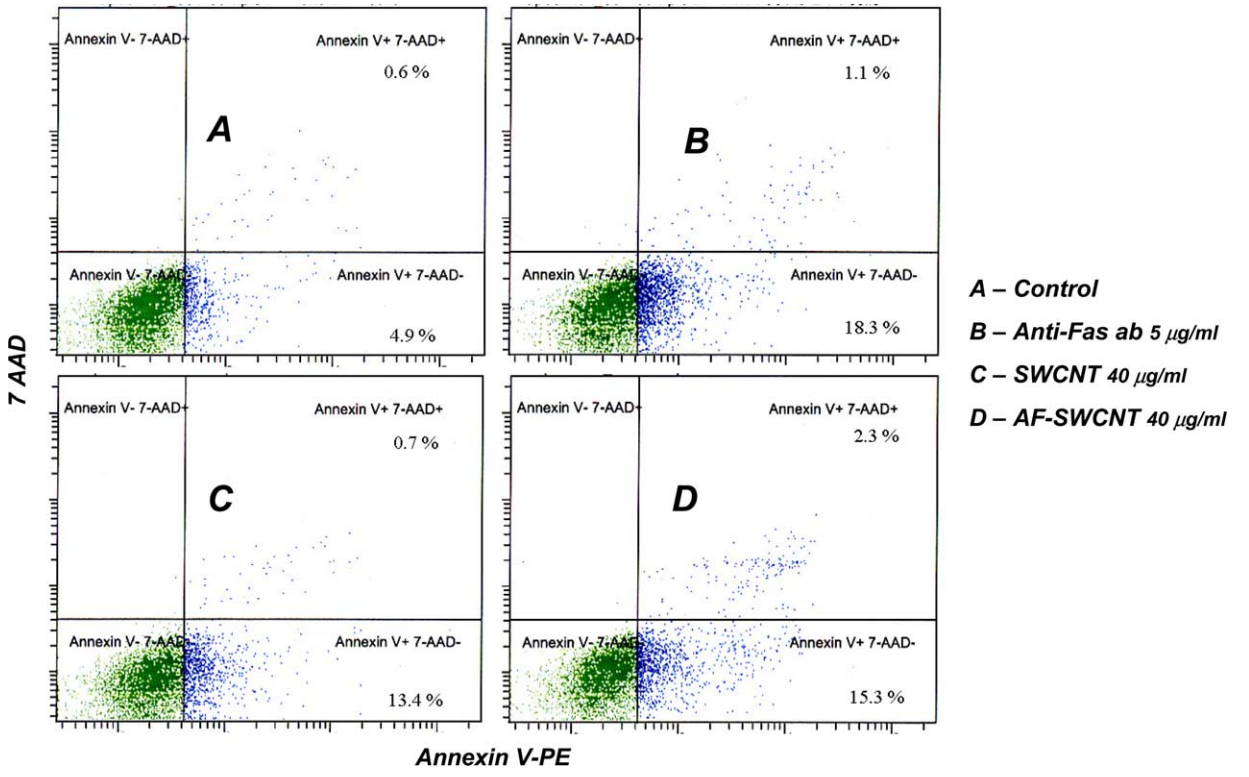

Figure 4. Apoptotic response of LA4 cells to control and acid functionalized SWCNTs. LA4 cells were cultured in 25 cm culture flasks with or without SWCNT, AF-SWCNT and anti-FAS antibody at given concentrations. After 24 h, cells were isolated by trypsinization, stained with 7AAD and Annexin V and analyzed by flow cytometry. Values in right quadrangles in each histogram indicate the percentage of dead ($7AAD^+$ Annexin V^+) and apoptotic ($7AAD^-$ Annexin V^+) cells in control and treated cell preparations. Results of a representative experiment are shown.

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neutralized the negatively charged AF-SWCNT particles treatment with poly L-lysine (PLL), a polymer of positively charged lysine amino acid. Pretreatment of AF-SWCNTs with PLL resulted in neutralization of the negative charge on AF-SWCNTs as indicated by a drop in zeta potential from −57.2 mV to −3.7 mV (Figure 5A) and a concomitant increase in average particle size from 137 nm to 2162 nm (Figure 5B). The effect of PLL pre-treatment of AF-SWCNT on its inhibitory effect on LA4 cell recovery was examined at dose levels of 10 and 20 μg/ml. The results in Figure 6 shows that PLL pre-treatment caused a significant drop in the otherwise potent inhibitory effect of AF-SWCNTs, but had no effect on the relatively poorer inhibitory effects of control SWCNT.

Pulmonary toxicity by oropharyngeal aspiration of SWCNT and AF-SWCNT

The toxic effects of SWCNT and AF-SWCNT were examined after oro-pharyngeal aspiration of 40 μg of SWCNT or AF-SWCNT in CD-1 mice with broncho-alveolar lavage (BAL) collected 18 and 72 h post-exposure. The results show that SWCNT had a modest effect on BAL cell count, BAL protein and LDH concentration, accumulation of PMNs and secretion of TNFα, IL6 and MIP2 cytokines (Figure 7). The AF-SWCNT treatment however caused a much more substantial increase in BAL cell count, protein concentration and PMN accumulation at the 18 h time point. A marked cytokine response to AF-SWCNT was also seen especially for IL6 and MIP2 secretion. The PMN accumulation declined at the 72 h time point though the overall cell count remained high as a result of a corresponding increase in macrophages (results not shown). The MIP-2 concentration remained elevated at 72 h while the IL6 titers and TNF-α levels declined toward control levels at this later time point. Overall, the results indicated that AF-SWCNT induced a significantly more potent inflammatory response in the lungs than the pristine SWCNTs.

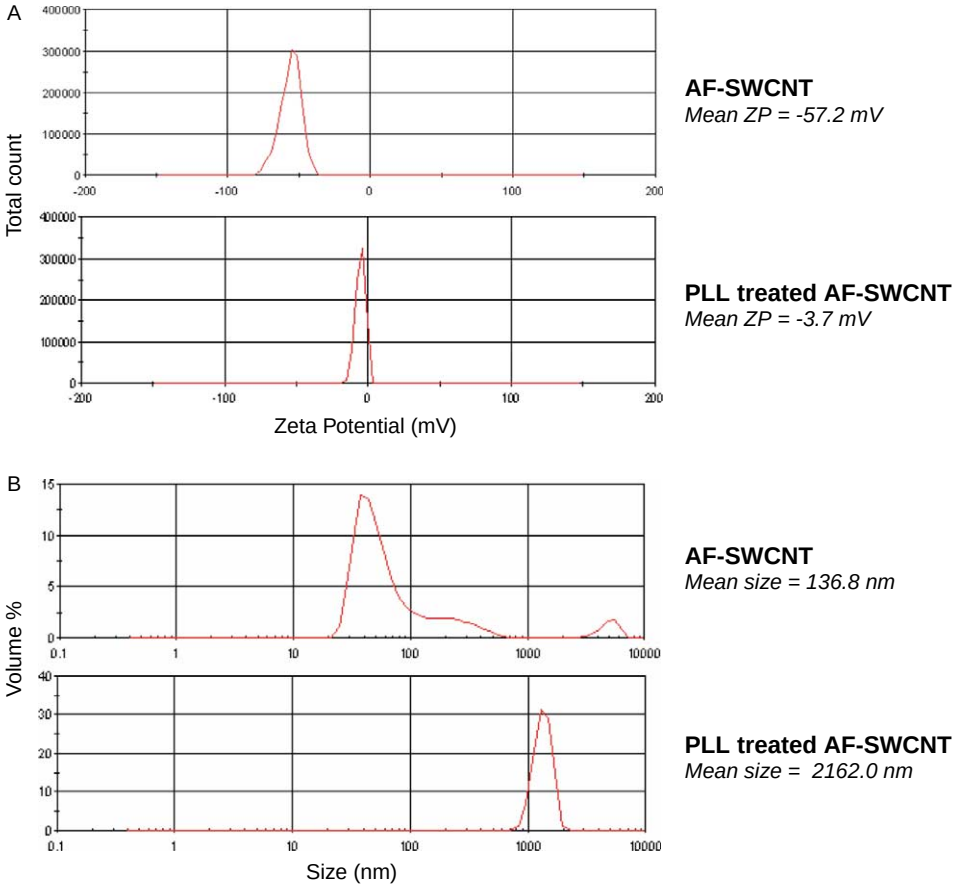

Figure 5. Charge and size distribution of AF-SWCNTs with and without treatment with poly-L lysine (PLL). Ten μl aliquots of SWCNT and AF-SWCNT preparations (2 mg/ml) were incubated with equal volume of PLL (2 mg/ml) for 1 hour at room temperature followed by dilution with 1 ml water before they were analyzed for size and charge using a Zetasizer Nano instrument. Although PLL treated AF-SWCNTs tended to agglomerate but they were still far better dispersed than pristine SWCNTs.

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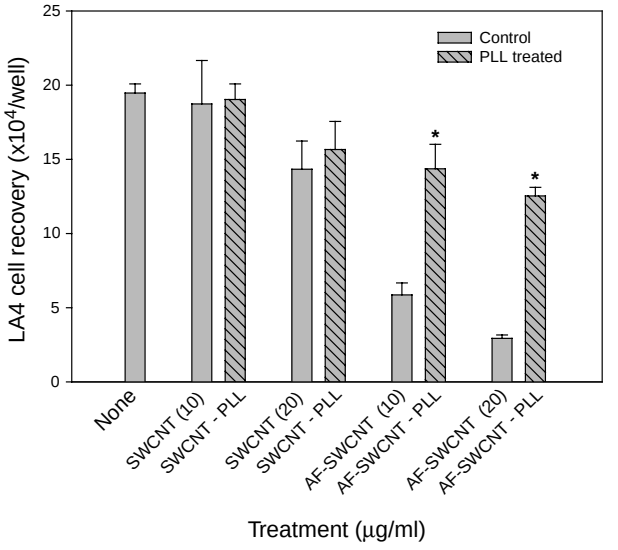

Figure 6. Effect of treatment with poly-L lysine (PLL) on the toxicity of AF-SWCNT preparations. AF-SWCNT preparations (2 mg/ml) were incubated with equal volume of PLL (2 mg/ml) for 1 h at room temperature. The incubation mixture was diluted 100-fold with culture medium to test the effects on LA4 cell recovery in a 3 days exposure paradigm described in legend to Figure 2. Results of a representative experiment are shown. All values are mean ± SD of four observations. *$p < 0.01$ as compared to PLL non-treated particles.

Discussion

Growing interest in developing diverse types of nanomaterials like SWCNTs for broad ranges of manufacturing and biomedical applications has necessitated studies of their potential toxicity in biological systems. To what extent the state of agglomeration and dispersion of carbon nanotubes influences their toxicity is however not known. It may be argued that the highly agglomerated SWCNT preparations may induce low or moderate toxicity due to limited bioavailability. Alternatively, it is also possible that the highly dispersed SWCNTs may not be toxic but become so upon agglomeration. These possibilities are difficult to test because pristine SWCNTs do not disperse well in aqueous media. Acid functionalized SWCNTs are readily solubilized in aqueous media but the impact of such chemical modifications on toxicity has not been explored. This study was designed to determine if acid-functionalization of SWCNTs that produces a highly dispersed product with high surface charge, also alters the toxic properties of SWCNTs.

Visual inspection showed that the unmodified SWCNTs agglomerated strongly in aqueous media and consistent suspensions could not be obtained even after vigorous point sonication. The AF-SWCNT dispersed to a much greater extent in aqueous solution with the bulk of the particles (98%) ranging between 25 and 152 nm. These results indicate that the process of acid functionalization did not destroy the nanotubes but caused significant disaggregation. Examination of the control and acid functionalized SWCNTs under

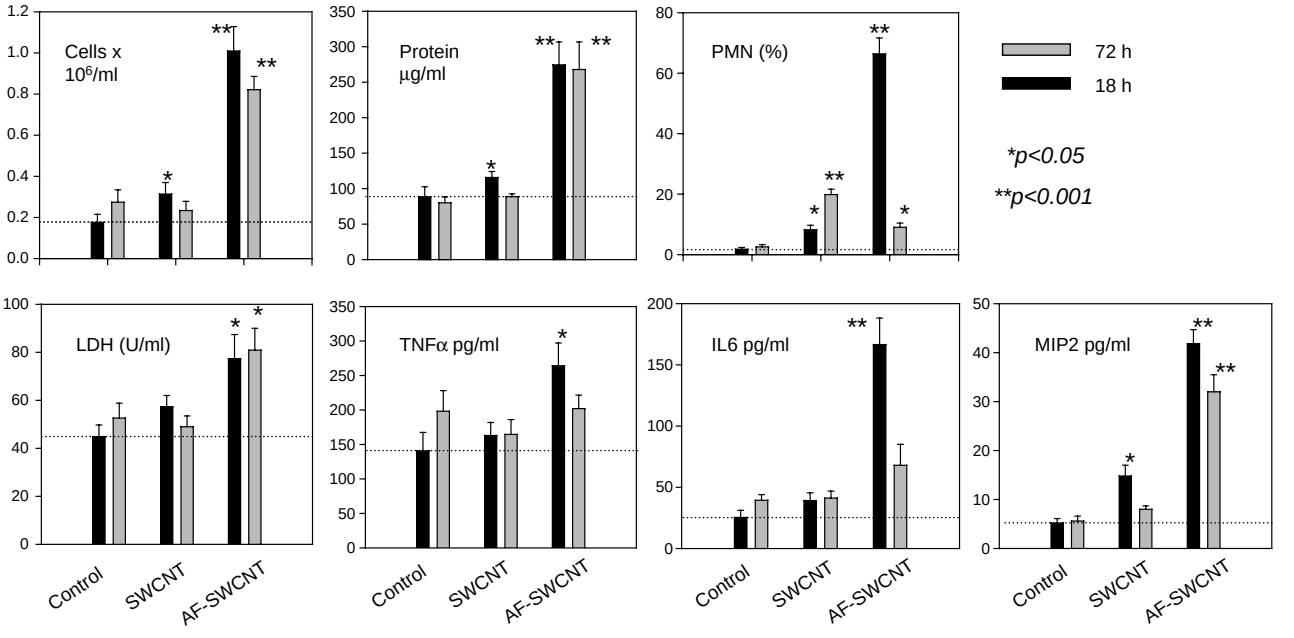

Figure 7. Pulmonary inflammatory response to control and acid functionalized SWCNT preparations. CD1 mice (mean bodyweight 31 ± 2.6 g) aspirated 50 μl normal saline containing 40 μg of SWCNT or AF-SWCNT. Control mice received saline alone. Eighteen and 72 h after the instillation, BAL fluid was collected and analyzed for various parameters of pulmonary inflammation. Results of a representative experiment have been shown. Each value represents mean ± SD of responses from four mice.

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scanning electron microscope further confirmed this point since the fine structure suggestive of bundled nanotubes were clearly seen in lyophilized AF-SWCNT preparations. As a consequence of the introduction of COO^- and $SO3^-$ groups AF-SWCNT particles were highly negatively charged. According to the ZetaSizer/Nano manual (www.Malvern.com), the zeta potential exclusion zone for stable particle suspensions is between +30mV and −30mV. The AF-SWCNTs particles had a ZP less than −40 mV suggesting they were stable in aqueous media.

Treatment with AF-SWCNTs resulted in a steep decline in the recovery of viable LA4 lung epithelial cells from cultures. Pristine SWCNTs also showed similar effect though the magnitude of the effect was much lower. Increased surface area and surface charge have also been identified as an important determining factor in the ability of particulates to interact with cells (Oberdörster 1996; Veronesi et al. 2002). The improved interaction between highly charged and dispersed AF-SWCNT preparations and LA4 cells could be the reason for a more potent effect of these preparations. Since better dispersion results from a greater charge on AF-SWCNT particles, it is not possible to state whether the enhanced potency of AF-SWCNT preparations was due to high charge or improved bioavailability. We attempted to neutralize the negative charge associated with AF-SWCNTs by treatment with the positively charged polymer, poly-L lysine. While PLL treatment reduced the charge on AF-SWCNT particles and partially neutralized the biological effect, it also resulted in re-agglomeration of particles. Thus the relative contributions of charge and surface area of SWCNT particles on their inhibitory effect on epithelial cells cannot be separated. The results do however show that the better dispersion resulting from high negative charge on carbon nanotubes enhances particle/cell interactions which could, (along with the presence of functional groups), contribute to the enhanced toxicity.

Further investigation into the mechanism of toxicity with cell cycling studies indicated that only 1.6% of the AF-SWCNT treated LA4 cells were in DNA synthetic S-phase whereas control and SWCNT treated cells had over 13% cells in S-phase. A decrease in the G2/M phase with a concomitant increase in the G1/G0 phase was also observed, and was the expected consequences of a block in S-phase (Shapiro 1988; Goya et al. 1993). In contrast, both SWCNTs and the AF-SWCNTs induced comparable levels of apoptosis compared to untreated controls. These observations suggest that while unmodified carbon nanotubes can by themselves induce apoptosis, the AF-SWCNTs also affect cell cycling thereby increasing the overall toxicity. A possible reason for this difference could be that the apoptotic pathways are triggered by interactions of pristine as well as acid-functionalized nanotubes through membrane interactions alone, whereas inhibition of the cell cycle requires internalization of the better dispersed AF-SWCNTs.

The *In vivo* efficacies of SWCNT and AF-SWCNT preparations to induce pulmonary toxicity in mice paralleled the *in vitro* results. The SWCNT preparation induced a weak inflammatory response as shown by a small but significant increase in BAL cell count, PMN infiltration and MIP2 secretion at the 18 h time point. In comparison, AF-SWCNT induced relatively stronger inflammatory responses that for some endpoints persisted even at 3 day time point.

In summary, SWCNTs caused modest but significant *in vitro* and *in vivo* toxicity, which is in agreement with several other published studies (reviewed in Lam et al. 2006). Acid- functionalized SWCNTs however were markedly more toxic in both *in vitro* and *in vivo* assays. This higher toxicity could result either from a possible greater bioavailability of well dispersed AF-SWCNT preparations, or from the high negative charge on AF-SWCNTs, or both. Due to the unique properties of carbon nanotubes, their applications in engineering and biomedical fields are expected to grow rapidly. Many such applications may warrant functionalized and charged nanotubes. The results of this study show that such chemical modifications could impart significantly higher toxicity to the parent materials. Handling of charged and dispersed nanotubes should be regarded as significantly more toxic by all routes of exposure until proven otherwise.

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