14

Critical Issues in the Evaluation of Possible Adverse Pulmonary Effects Resulting from Airborne Nanoparticles

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INTRODUCTION

Nanotechnology is the manipulation of matter on a near-atomic scale to produce new structures, materials, and devices. Engineered nanoparticles are defined as having at least one dimension <100 nm. Because of their small size, nanoparticles have a high particle surface area/mass and exhibit physicochemical properties that differ dramatically from fine-sized particles of the same composition. These unique properties are being exploited for a number of applications, including integrated sensors, semiconductors, structural materials, drug delivery systems, medical imaging, sunscreens, cosmetics, and coatings (1). Therefore, nanotechnology has the ability to transform many industries from manufacturing to medicine. By 2015, the National Science Foundation estimates that nanotechnology will have a $1 trillion impact on the global economy and employ 2 million workers, 1 million of which may be in the United States.

Since nanoparticles are engineered to exhibit unique physicochemical properties, it would be reasonable to expect that these nanoparticles would interact with biological systems in ways that may be dramatically different from fine-sized particles of the same composition. The Royal Society and

Royal Academy of Engineering recognized the challenge of predicting whether exposure to nanoparticles would be a health concern, which routes of entry should be avoided, and which nanoparticle exposures should be controlled (2).

If nanoparticles become airborne during production or use, the effects of pulmonary exposure require evaluation. Thus, far little information is available concerning airborne levels of nanoparticles in the nanotechnology industry. Maynard et al. (3) reported that respirable airborne dust levels in laboratory settings producing single-walled carbon nanotubes (SWCNT) by either a high-pressure carbon monoxide (HiPCO) or laser ablation process were generally low, 53 $\mu g/m^3$. However, peaks were noted during certain handling processes. In addition, laboratory studies indicate that airborne levels of SWCNT can be increased significantly by agitation, i.e., using a vortex shaker or a fluidized bed generator. Therefore, airborne levels in nanotechnology workplaces would depend on the energetics of the processes involved during production and use as well as the presence of control systems.

Given that aerosolization of nanoparticles is possible, adverse respiratory effects are a concern. This chapter will review some properties of nanoparticles, which are critical issues for investigation of pulmonary toxicology. These issues include: deposition, interstitialization, translocation, role of surface area, and role of oxidant stress in the pulmonary toxicity of nanoparticles.

Deposition

For a given particle, pulmonary response is dependent on the fraction of inhaled particles, which remain deposited in the lung. Deposition fraction is dependent on the aerodynamic diameter of the particle (4–6). Deposition models predict that deposition of coarse (>2.5 μm) and fine (0.1–2.5 μm) particles is governed by the processes of sedimentation and impaction. In the case of nanoparticles (<100 nm), particle mass and momentum are extremely small; thus, sedimentation and impaction are not significant factors in pulmonary deposition. Rather, nanoparticles behave like gas molecules, moving randomly by Brownian motion. Such movement would result in the random contact of nanoparticles with the epithelial and/or fluid lining the lung. Models predict that the deposition of nanoparticles in both the tracheobronchial and pulmonary regions of the lung can far exceed that of fine or coarse particles (4–6). In addition, as nanoparticles become smaller than 10 nm, nasal deposition by diffusional mechanisms becomes very high.

Experimental data are available supporting the high deposition of inhaled nanoparticles in the lung. Kreyling et al. (7) exposed rats ventilated via an endotracheal tube by inhalation to radioactive iridium particles (^{192}Ir)

with a primary particle size of either 15 or 80 nm. They reported thoracic deposition fraction (deposited in the tracheobronchial plus the pulmonary regions) to be 49% and 28% for the 15 or 80 nm nanoparticles, respectively. Daigle et al. (8) conducted a deposition study in humans upon inhalation of 8.7 or 26 nm carbon particles. They reported that deposition fraction for the respiratory tract was 66% and 80% for 8.7 or 26 nm particles, respectively, at rest. Deposition fraction increased significantly to 83% or 94%, respectively, upon exercise.

In conclusion, both deposition models and experimental results indicate that a high fraction of inhaled nanosized particles can deposit in airways of the conducting and respiratory zones of the lungs. This deposition of nanosized particles is much higher than lung burdens expected after inhalation of equivalent amounts of coarse or fine particles. These relatively high lung burdens are an area of concern when considering the potential pulmonary toxicity of a nanoparticle.

INTERSTITIALIZATION

Since a relatively high percentage of inhaled nanosized particles are likely to deposit on the alveolar surfaces, an issue is whether nanosized particles are effectively phagocytized and cleared by alveolar macrophages or whether a significant number of particles enter the alveolar interstitium where they may cause interstitial damage, inflammation, and/or fibrosis. Results from Kreyling and Scheuch (9) indicate that upon pulmonary exposure fine particles are more effectively phagocytized and cleared by alveolar macrophages than nanosized particles. The fact that a fraction of nanosized particles may avoid phagocytosis results in a greater interstitial burden of nanosized TiO_2 than with an equivalent mass exposure of fine-sized TiO_2 (10). A careful investigation of the interstitialization of fine versus nanosized particles was reported by Oberdorster et al. (11). In this study, rats were exposed to nanosized (20 nm) or fine (250 nm) particles by inhalation for 12 weeks at respective concentrations that resulted in a similar mass deposition of the two particle types. The amount of nanosized TiO_2 in the alveolar interstitium and lymph nodes significantly exceeded the amount of fine TiO_2 in these sites at all time points studied over a 1-year postexposure period. At 1-year postexposure, 44% of deposited nanosized TiO_2 had migrated to the interstitium compared to 13% for fine TiO_2.

In a recent investigation, Roberts et al. (12) exposed rats by intratracheal instillation to fluorescently labeled quantum dots (30–50 nm) and monitored the deposition and fate of these nanoparticles from 2 h to 5 days postexposure. At 2 h postexposure, some quantum dots were located in the alveolar airspaces and on the alveolar epithelial surface, while other quantum dots had already been phagocytized by alveolar macrophages.

At 24 h postexposure, most quantum dots were found within alveolar macrophages. However, a small fraction of deposited quantum dots had avoided phagocytosis and could be found in the alveolar interstitium after 24 h. It appears that the fraction of deposited nanoparticles that are phagocytized can vary greatly with particle type. Unlike quantum dots, which were avidly phagocytized by alveolar macrophages both in vitro and in vivo (12), SWCNT appear to be poorly phagocytized by macrophages in culture (13). Mercer et al. (14) evaluated phagocytosis and interstitialization of aspired SWCNT, which had been labeled with colloidal gold nanoparticles. Only a small fraction of deposited gold-labeled SWCNT was engulfed by alveolar macrophages, while most of the SWCNT were rapidly incorporated into the alveolar interstitium. In contrast, aspiration of gold nanoparticles resulted in avid phagocytosis by alveolar macrophages with only a few gold particles in the interstitium.

In conclusion, not only are nanoparticles highly deposited in the alveoli, but particle number/mass is so great that some nanoparticles escape phagocytosis by alveolar macrophages and can enter the alveolar interstitium. The recognition of nanoparticles by alveolar macrophages is dependent on particle type, i.e., quantum dots and nanogold particles are rapidly phagocytized by alveolar macrophages while SWCNT are not. Therefore, a large fraction of deposited SWCNT rapidly migrates to the alveolar interstium. This high interstitial deposition of SWCNT after aspiration has been associated with diffuse interstitial fibrosis of rapid onset, which progresses over 60 days postexposure (13).

TRANSLOCATION

If a large fraction of inhaled nanoparticles can deposit in the alveoli and a significant number of these nanoparticles can escape phagocytosis and enter the alveolar interstitium, an issue is whether nanoparticles within the alveolar walls can enter pulmonary capillaries and translocate to systemic sites. A study by Oberdorster et al. (15) involving whole body inhalation of ^{13}C nanoparticles (20–24 nm) in rats indicated that substantial numbers of ^{13}C nanoparticles were found in the liver within 18 h postexposure. Relatively rapid translocation of nanoparticles from the lung to the blood was also reported in hamsters after inhalation of nanosized carbon labeled with technetium (16). Similarly, Stone and Godleski (17) reported migration of nanosized particles in the liver and heart following inhalation exposure. In contrast to the reports above, Kreyling et al. (7) investigated translocation of ^{192}Ir (15 or 80 nm) and reported than 1% of these nanoparticles given by inhalation via an endotracheal tube were found in the liver between 6 h and 2 days postexposure. Kreyling and colleagues (7) explained these disparate results by suggesting that the Oberdoster et al. (15) study involved

whole body exposure. Therefore, oral exposure during grooming and absorption of particles via the gastrointestinal tract was possible. They also argued that the technetium label may have dissociated from the carbon nanoparticles and entered the blood.

In conclusion, nanoparticles can enter the alveolar interstitium. Once in the interstitium they can migrate into pulmonary capillaries and translocate to systemic sites. At present, the rate of this translocation is a matter of debate and requires further investigation. Investigations are currently in progress at the National Institute for Occupational Safety and Health to quantify the extent of translocation of gold-labeled SWCNT or metal oxide nanoparticles following pulmonary exposure by monitoring the metal content of systemic organs by neutron activation analysis. If nanoparticles translocate from the lungs to systemic organs, then the possible adverse effects of inhalation of nanoparticles on the function of these organs requires investigation. Indeed, there is evidence that aspiration of SWCNT can cause oxidant stress in cardiac and aortic tissue and augment plaque formation in an atherosclerotic susceptible mouse model (18–20).

ROLE OF SURFACE AREA IN TOXICITY

Nanoparticles are characterized by a high surface area per mass. A current hypothesis is that particulate surface area and available surface-active sites play an important role in the biological activity of a particle. Support for this hypothesis can be provided from a simple experiment to evaluate the cytotoxicity of crystalline silica particles of different diameters on human alveolar type II epithelial cell line (A549) in culture (Fig. 1). In this experiment, surface area of SiO_2 (<2 μm) was 5.90 ± 0.03 m^2/g while SiO_2 (<10 μm) was 1.75 ± 0.03 m^2/g, as measured by gas absorption (BET analysis). When exposure dose was on an equivalent mass basis (μg/mL), the smaller silica particles with the greater surface area were 1.9-fold more cytotoxic than the larger silica particles at 70 μg/mL (Fig. 1A). However, when dose was normalized to equivalent particle surface area/cell surface area, there was no difference in the toxicity of the two sizes of silica (Fig. 1B). Similar results were reported for exposure of a human alveolar type II epithelial cell line (A549) to fine or ultrafine TiO_2 and stimulation of message for IL-8. When exposure dose was normalized to equivalent particle surface area, both fine and ultrafine TiO_2 were equipotent in induction of mRNA for this chemokine (21). This dependence of biological activity on particle surface area has also been demonstrated in vivo (22). In this study, rats were exposed to fine (250 nm) or ultrafine (20 nm) TiO_2 particles by intratracheal instillation and pulmonary inflammation determined by the number of polymorphonuclear (PMN) leukocytes harvested by bronchoalveolar lavage

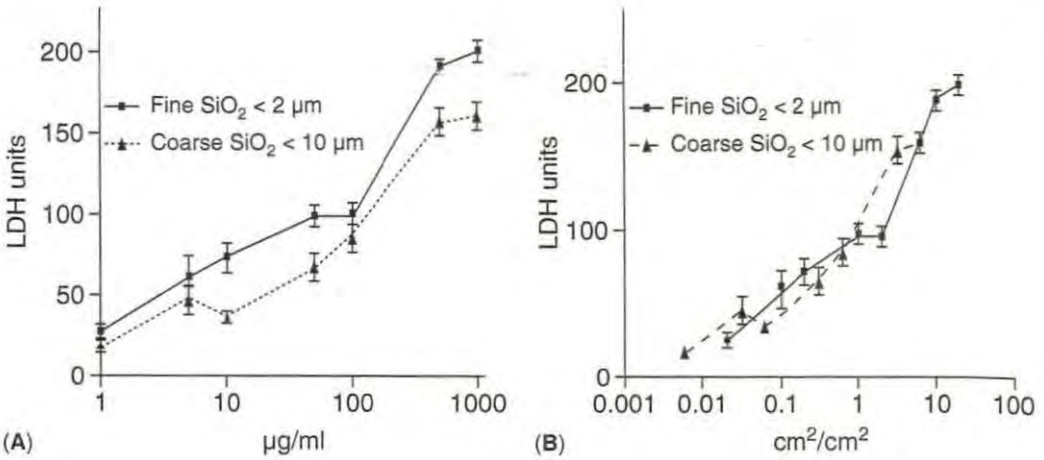

Figure 1 Size dependence of silica cytotoxicity. Human alveolar type II epithelial cells (A549; 1.5×10^5 cells/mL/well) were exposed to various concentrations of crystalline silica of two particle sizes (<2 μm; surface area = 5.9 m^2/g or <10 μm, surface area = 1.75 m^2/g). Cytotoxicity was measured as the leakage of lactate dehydrogenase (LDH) into the medium. (**A**) Response versus dose on an equivalent mass/mL basis. (**B**) Response versus dose on an equivalent particle surface area/cell surface area basis. Data are means ± SE of three experiments.

(BAL) at 24 h postexposure. On an equivalent mass basis, ultrafine TiO_2 was more inflammogenic than fine TiO_2 (1.9-fold more potent at 500 μg/rat). However, when Oberdorster (22) normalized exposure dose to equivalent surface area, the inflammatory potency of ultrafine and fine TiO_2 was similar. A similar relationship between particle surface area and inflammatory potential or depression of particle clearance was reported after inhalation exposure to fine versus ultrafine TiO_2 (11).

Nanoparticles have a tendency to agglomerate into μm-sized particles when suspended in saline. If particle surface area is critical to pulmonary response, would the presence of agglomerates affect the biological responses of cells or the lung to in vitro or in vivo (intratracheal instillation or pharyngeal aspiration) exposure to suspensions of nanoparticles? As shown in Figure 2A, suspension of ultrafine carbon black (Printex 90, Degussa Corporation, Parsippany, New Jersey, U.S.; 14 nm primary particle diameter) in phosphate-buffered saline (PBS) resulted in μm-sized agglomerates even after sonication. Since inhaled nanoparticles would come in contact with alveolar lining fluid, we hypothesized that one could collect diluted alveolar lining fluid by a single BAL of rat lungs and use this BAL fluid to suspend nanoparticles (23,24). Suspension of ultrafine carbon black in acellular BAL fluid resulted in a much improved dispersion of ultrafine carbon black with many structures in the submicrometer size (Fig. 2B). Better dispersion of nanoparticle suspensions had a dramatic effect on the biological activity of ultrafine carbon black in rat lungs 24 h after intratracheal instillation. Pulmonary exposure to ultrafine carbon black

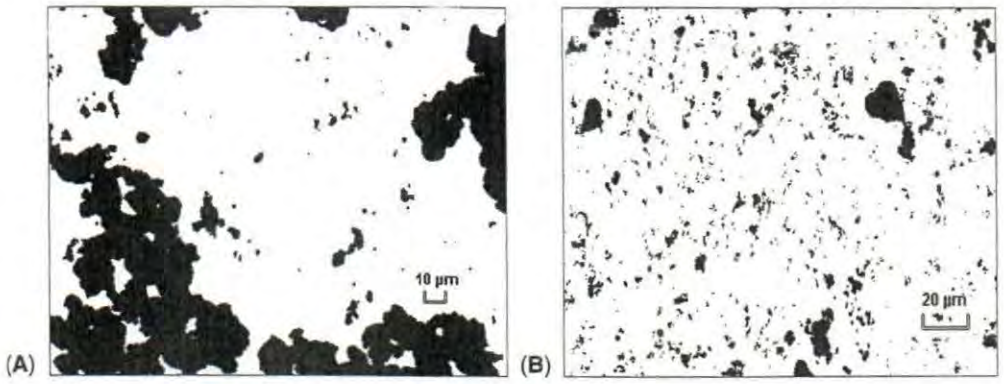

Figure 2 Dispersion of ultrafine carbon black. Ultrafine carbon black (Printex 90, 14 nm) was suspended in media and briefly sonicated (5 individual pulses) then viewed under a light microscope at ×40. (**A**) Suspension in PBS showing μm-sized agglomerates. (**B**) Suspension in bronchoalveolar lavage fluid, i.e., diluted lung lining fluid, showing substantially improved dispersion. *Abbreviation*: PBS, phosphate-buffered saline.

suspended in PBS caused a dose-dependent inflammatory response (PMN infiltration) 24 h postexposure (Fig. 3A). However, ultrafine carbon black suspended in BAL fluid was significantly more potent at doses ranging from 0.047 to 1.5 mg/rat. Maximum inflammation at 1.5 mg/rat was twofold greater for nanoparticles suspended in BAL fluid with an equivalent level of inflammation seen at a 16-fold lower dose of the well-dispersed carbon black compared to the nanoparticles suspended in PBS. Likewise, exposure of rat lungs to ultrafine carbon black suspended in PBS caused dose-dependent lung damage as indicated by elevated lactate dehydrogenase (LDH) activity in the BAL fluid (Fig. 3B). However, particles dispersed in BAL fluid were more cytotoxic at all exposure doses. Maximum cytotoxicity was 1.7-fold greater in the BAL fluid-dispersed nanoparticles with an equivalent level of toxicity seen at a 16-fold lower dose.

Improved dispersion of nanoparticles also alters the character as well as the magnitude of pulmonary response. Pharyngeal aspiration of SWCNT to mice resulted in granulomatous lesions at deposition sites of large agglomerates and interstitial fibrosis at sites containing more dispersed SWCNT structures (13). Mercer et al. (14) reported that when efforts are made to improve the dispersion of SWCNT the mean diameter of SWCNT agglomerates can be significantly decreased. In contrast to the initial study by Shvedova et al. (13), pharyngeal aspiration of mice with dispersed SWCNT did not result in granulomas while the potency in initiating diffuse interstitial fibrosis increased by at least fourfold (14).

In conclusion, there is support for the hypothesis that the high surface area of nanoparticles plays a significant role in their bioactivity. Evidence also exists that efforts to improve the dispersion of nanoparticles and decrease the size of agglomerates delivered to in vitro or in vivo test systems

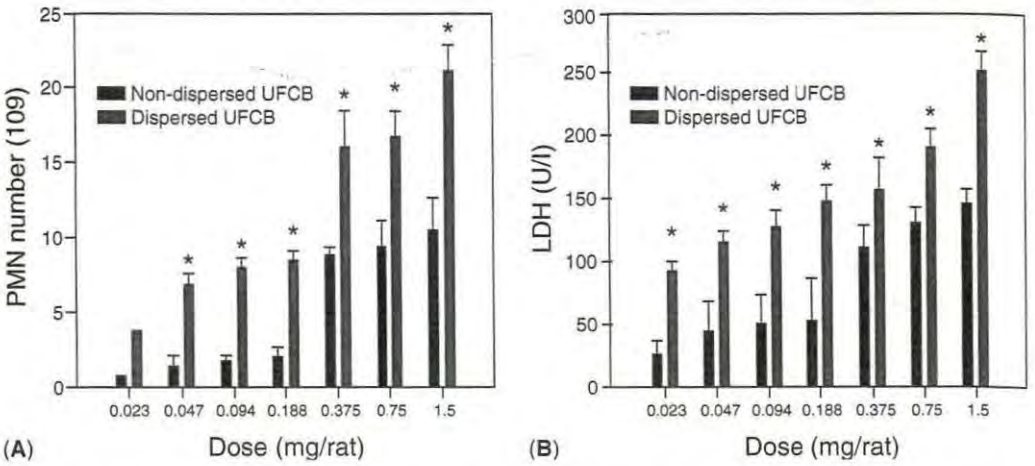

Figure 3 Pulmonary response to exposure to ultrafine carbon black. Rats were exposed to ultrafine carbon black by intratracheal instillation. (**A**) Inflammation was monitored by BAL PMN counts 24 h postexposure. (**B**) Toxicity was monitored by BAL LDH activity 24 h postexposure. Values are means ± SE of 5 experiments. * indicates that particles dispersed in BAL fluid produce a significantly greater pulmonary response than particles suspended in PBS. *Abbreviations*: BAL, bronchoalveolar lavage; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PMN, polymorphonuclear.

increases the magnitude of response, and in the case of SWCNT alters the site and character of the pulmonary response. Therefore, efforts should be made to increase the dispersion of nanoparticles in the delivery vehicle for in vitro and in vivo exposure. Sager et al. (23,24) demonstrated the effectiveness of BAL fluid in dispersing nanoparticles and have reported that a mixture of disaturated phosphatidylcholine (DSPC) and protein, at levels found in BAL fluid, was also effective.

ROLE OF OXIDANT STRESS IN TOXICITY

Nel et al. (25), in a recent review of nanotoxicology, noted that oxidant generation by nanoparticles and resultant oxidant stress to cells is the "best developed paradigm for nanoparticle toxicity." Shvedova et al. (26) conducted a study on the effects of exposure of human bronchial epithelial cells (BEAS-2B) to unpurified SWCNT containing 30% iron by mass. Unpurified SWCNT-generated oxidant species in a cell-free system and hydroxyl (OH) radical in the presence of BEAS-2B cells, which served as a source of H_2O_2 to induce a Fenton reaction. Unpurified SWCNT were cytotoxic to BEAS-2B cells and addition of the iron chelator, deferoxamine, reversed both radical generation and cytotoxicity. The radical generation by unpurified SWCNT was associated with depletion of cell antioxidants and enhancement of lipid peroxidation in BEAS-2B cells, indicating that oxidant stress had occurred.

To further evaluate the hypothesis that oxidant generation and resultant oxidant stress were predictors of cytotoxicity, BEAS-2B cells were exposed to three types of engineered carbon nanoparticles, i.e., fullerenes, SWCNT produced by the HiPCO process, and SWCNT, i.e., LM-90, produced by the laser ablation method. The HiPCO SWCNT sample contained 30% iron by mass, while the LM-90 sample contained 20% nickel by mass. In these studies, the carbon nanoparticles were suspended in PBS and dispersed by sonication prior to use. Figure 4 shows TEM micrographs of HiPCO SWCNT (A), fullerenes (B), and LM-90 (C) in suspension. HiPCO SWCNT structures were loose networks of nanotubes with iron nanoparticles (arrows) bound to the nanotubes. LM-90 structure also appeared to be loose networks of nanotubes in suspension with nickel nanoparticles (arrows) bound to the nanotubes. Fullerenes formed agglomerates of widely variable diameters in suspension. Suspension of the carbon nanoparticles in PBS containing 1 mM H_2O_2 and 100 mM DMPO as a spin trap resulted in the generation of ·OH radical measured by electron spin resonance (ESR) spectroscopy. From the peak heights, the

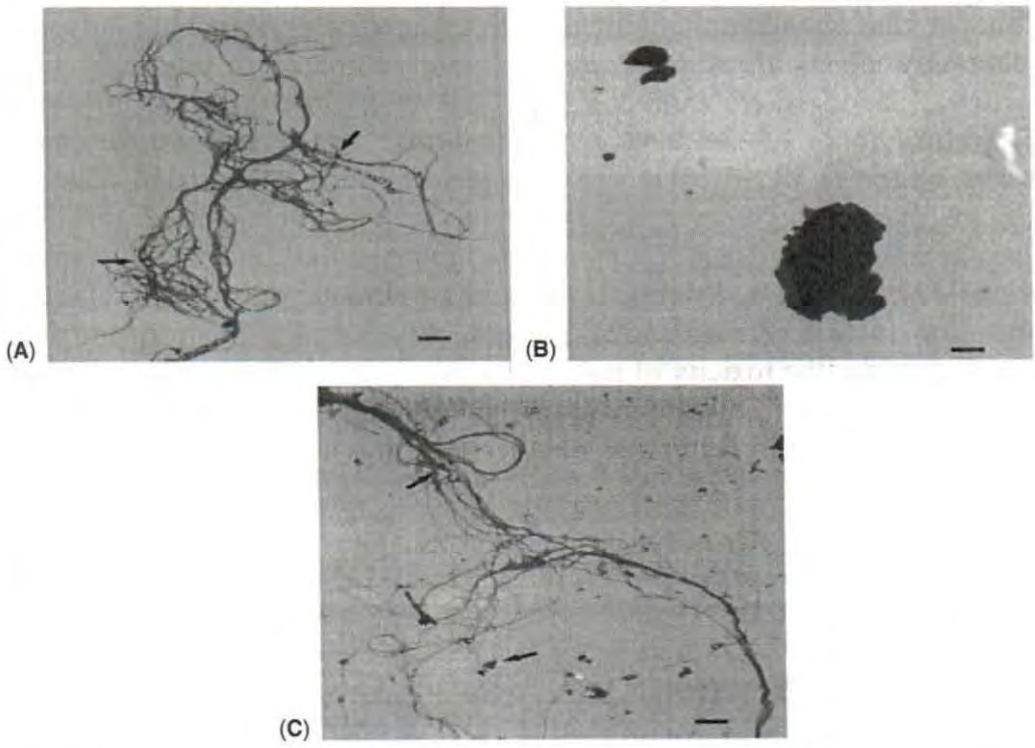

Figure 4 Structure of carbon nanoparticles in suspension. Particles were added to distilled H_2O and sonicated. Morphology of HiPCO SWCNT (**A**), fullerenes (**B**), or LM-90 (**C**) under TEM. Arrows indicate adherent iron (**A**) or nickel (**C**) nanoparticles identified by EDX. Scale bars are 200 nm. *Abbreviations*: EDX, HiPCO SWCNT, high-pressure carbon monoxide single-walled carbon nanotubes; TEM, transmission electron micrograph.

potency sequence for ·OH generation was fullerenes >LM-90 > HiPCO SWCNT (Table 1). Exposure of BEAS-2B cells to these carbon nanoparticles (8.5 μg/cm^2) caused oxidant stress, measured as a decline in cellular levels of glutathione (GSH). The potency sequence was HiPCO SWCNT ≈ LM-90 > fullerenes (Table 1). Exposure of BEAS-2B cells to these carbon nanoparticles (8.5 μg/cm^2) caused a significant decrease in cell viability. The potency sequence was HiPCO SWCNT > fullerenes > LM-90 (Table 1).

In summary, the data indicate that carbon nanoparticles can generate reactive oxygen species, which results in oxidant stress and cytotoxicity. However, the relationship among these events is not simple in that radical generation of three types of carbon nanoparticles did not directly predict the degree of oxidant stress and these events did not directly predict the degree of cytotoxicity (Table 1). Therefore, other characteristics, such as agglomerate size or density of the agglomerates, may also contribute to bioactivity, interaction with the cell membrane, and/or uptake into the cells.

CONCLUSION

Concern that inhalation of engineered nanoparticles may have adverse pulmonary effects arises from the fact that, compared to coarse or fine particles, nanoparticles exhibit a high deposition in the conducting and respiratory zones of the lung. Once deposited, some nanoparticles may escape clearance by alveolar macrophages and enter the alveolar interstitium. There is some evidence that nanoparticles can migrate from the alveolar interstitium to the pulmonary capillary blood and translocate to systemic organs. Thus, systemic toxicity after pulmonary exposure is also an issue for further research. At present, two of the most promising mechanisms for the toxicity of nanoparticles involve their high surface area and the ability to generate reactive species and cause oxidant injury. Research is needed to determine whether these mechanisms are universally

Table 1 Effect of Different Nanoparticles on Viability, Glutathione Levels, and Oxidant Generation in BEAS-2B Cells (8.50 μg/cm^2; 18 h)

Particle	Viability % of control	GSH % of control	·OH generation average peak height
Fullerenes	88.43 ± 1.42[a,b]	91.02 ± 1.63[a]	116 ± 6.821[b,c]
LM-90	93.664 ± 0.625[a]	71.35 ± 0.84[a,d]	68.0 ± 7.0[c]
SWCNT	84.232 ± 1.166[a,b,d]	68.75 ± 1.60[a,d]	42.50 ± 2.754

Note: Values are means ± SE of 3 experiments.
[a] $p < .05$ versus control.
[b] $p < .05$ versus LM-90.
[c] $p < .05$ versus control.
[d] $p < .05$ versus SWCNT.

predictive of cytotoxicity and pulmonary response. Clearly the effects of agglomeration, particle shape, particle coatings or surface groups, particle charge, etc. on bioactivity are issues requiring further investigation.

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