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Dispersion medium modulates oxidative stress response of human lung epithelial cells upon exposure to carbon nanomaterial samples

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Abstract

Due to their large specific surface area, the potential of nanoparticles to be highly reactive and to induce oxidative stress is particularly high. In addition, some types of nanoparticles contain transition metals as trace impurities which are known to generate reactive oxygen species (ROS) in biological systems. This study investigates the potential of two types of single-walled carbon nanotube samples, nanoparticulate carbon black and crocidolite asbestos to induce ROS in lung epithelial cells *in vitro*. Carbon nanotube and carbon black samples were used as produced, without further purification or processing, in order to best mimic occupational exposure by inhalation of airborne dust particles derived from carbon nanoparticle production. Intracellular ROS were measured following short-term exposure of primary bronchial epithelial cells (NHBE) and A549 alveolar epithelial carcinoma cells using the redox sensitive probe carboxydichlorofluorescein (carboxy-DCFDA). The oxidative potential of agglomerated nanoparticle samples was compared following dispersion in cell culture medium with and without foetal calf serum (FCS) supplement. In addition, samples were dispersed in dipalmitoylphosphatidylcholine (DPPC), the major component of lung surfactant. It could be illustrated that *in vitro* exposure of lung epithelial cells to carbon nanomaterial samples results only in moderate or low oxidative stress under the exposure conditions employed. However, cell responses are strongly dependent on the vehicle used for dispersion. Whereas the presence of DPPC increased intracellular ROS formation, FCS seemed to protect the cells from oxidative insult.

Keywords:

Nanomaterials, carbon black, carbon nanotubes, asbestos, reactive oxygen species, lung surfactant, DPPC

Introduction

Due to their unique electrical, physical and thermal qualities, carbon based nanomaterials, including carbon nanoparticles, nanofibers, single- and multiwalled carbon nanotubes (SWCNT and MWCNT) are currently one of the most attractive engineered nanomaterials offering a variety of potential future applications [Huczko *et al.*, 2002; The Royal Society, 2004]. In addition to industrial use, various biomedical applications of nanomaterials, including carbon nanotubes, have been proposed [Bianco *et al.*, 2005]; [Lacerda *et al.*, 2006]. This has led to increased global production of carbon nanotubes (CNT), resulting in a concomitant increase in the risk for human and environmental health, as some of the properties that render nanoparticles technologically attractive are also the cause for concern [Maynard *et al.*, 2004b; Oberdorster *et al.*, 2005b]. The high biopersistence and structural resemblance of CNT to asbestos has put CNT into a particularly critical position [Donaldson *et al.*, 2006]. The small size of CNT may not only allow them to gain access to cells and tissues, it also leads to large specific surface area which may render them highly reactive. Therefore, the risk of CNT to induce oxidative stress is particularly high.

The effects of nanoparticle exposure seen in animal studies indicate a role of oxidative stress in the production of inflammatory cytokines and cytotoxic cellular responses [Beck-Speier *et al.*, 2005; Donaldson *et al.*, 2006; Li *et al.*, 2002; Li *et al.*, 2008; Nel *et al.*, 2006; Oberdorster *et al.*, 2005a; Oberdorster *et al.*, 2005b]. Reactive oxygen species (ROS) react with a wide range of biological targets and are known to be involved in both cellular signaling and cell damage [Briviba *et al.*, 1997]. ROS generation through Fenton reactions or following phagocytosis has been directly related to fibre toxicity and has also

been associated with carbon nanotube (CNT) exposure, leading to lipid peroxidation, oxidative stress and changes in cell morphology [Sayes *et al.*, 2005; Shvedova *et al.*, 2003]. Other consequences of oxidative injury include effects on nuclear factor activation, gene transcription and protein [Shukla *et al.*, 2003; Vallyathan and Shi, 1997]. This study investigates the oxidative stress response following in vitro exposure to three types of carbon nanomaterials, high pressure carbon monoxide produced SWCNT (HiPco SWCNT), arc discharge produced SWCNT and P90 carbon black, following dispersion in biological media. As-produced nanomaterials are employed, unpurified and minimally processed, in order to best mimic occupation exposure by inhalation of airborne dust particles derived from carbon nanoparticle production. The dispersions thus consist of nanomaterial agglomerates. Furthermore, as produced samples of SWCNT contain variable amounts of catalyst residues, most of which are transition metals such as iron, cobalt and nickel. The type of impurities present depends on the type of catalysts used during production, and thus a comparison of different nanotube type is merited.

The lungs are one of the main potential exposure targets during the manufacture and processing of nanoparticles [Maynard *et al.*, 2004a; Shvedova *et al.*, 2003; Smart *et al.*, 2005]. Therefore, lung epithelial cells, including the alveolar type II carcinoma cell line A549 and normal human bronchial epithelial cells (NHBE), were chosen as target cells for this study. Within the lung, the bronchioles and alveoli are covered by pulmonary surfactant. The main physiological role of this lipid bi-layer is to allow efficient gas exchange by lowering the surface tension [Schurch *et al.*, 1992]. Inhaled particles may come into contact with this surfactant upon inhalation exposure which may lead to particle coating and modification of the surface chemistry [Wallace *et al.*, 2007a; Wallace

et al., 2007b]. In turn, this may have consequences for the oxidative potential of the particles. In addition, lung surfactant can influence particle dispersion [Herzog *et al.*, 2009] which would ultimately lead to changes in particle size distribution, agglomeration state or surface area, all factors that are believed to play an important role in the toxicity of carbon nanoparticles [Oberdorster *et al.*, 2005a].

Therefore, dispersions were carried out in the presence and absence of serum supplements and dipalmitoylphosphatidylcholine (DPPC) in order to assess the influence of dispersion vehicle on the potential of test materials to induce oxidative stress in human lung epithelial cells. The results are compared to those observed for crocidolite asbestos fibers.

Materials and Methods

Cell Culture

A549 human lung epithelium

A549 cells from a human lung adenocarcinoma with the alveolar type II phenotype were obtained from ATTC (Manassas, VA, USA). Cells were cultured in RPMI 1640 (Gibco, Karlsruhe) supplemented with L-glutamine, penicillin and streptomycin (Gibco) and 10% foetal calf serum (FCS, Biochrom, Berlin) in a humidified atmosphere containing 5% CO₂ at 37 °C. All cell culture reagents were obtained from PAA Laboratories (Pasching, Austria).

Normal human primary bronchial epithelial cells

Normal human primary bronchial epithelial cells (NHBE) were obtained from Clonetics™ (Lonza, Switzerland) and were maintained in bronchial epithelial cell basal medium (BEBM®) plus SingleQuots® supplements as recommended by the manufacturer. Cells were used between passages 3-7.

Test Particles

Carbon nanoparticles

HiPco derived single-walled carbon nanotubes (SWCNT) were purchased from Carbon Nanotechnologies, Inc. (Houston, TX) and contained 10 wt% iron catalyst residues. The diameter distribution of these nanotubes was previously determined by Raman spectroscopy and ranged between 0.8 – 1.2 nm [Hedderman, 2006]. Atomic force microscopy (AFM) revealed HiPco SWCNT in their “as produced” state to exist in

bundles on average 800 nm long with estimated bundle sizes of $2.6 \times 10^{14} \text{ m}^2$. A BET surface area of $487.15 \text{ m}^2/\text{g}$ was measured based on nitrogen adsorption. These particles were extensively described in a recent publication, since the exact same treatment was used for the current study, a more detailed particle characterization can be found in Herzog *et al.* (2009). Arc discharge synthesized SWCNT were obtained from Sigma Aldrich, product number 519308 (Dublin, Ireland) and contained 50 to 70 percent SWCNT, the principle impurities being amorphous carbon, turbostratic graphite and trace amounts ($< 1 \text{ wt}\%$) of nickel and yttrium catalysts. Tubes are reported to be 1.2 to 1.5 nm in diameter and 2 to 5 μm long, occurring in bundles of around 20 μm in length (Sigma Aldrich). AFM showed estimated bundle sizes of $4.1 \times 10^{-14} \text{ m}^2$ [Hedderman, 2006]. BET surface area was determined to be $239.47 \text{ m}^2/\text{g}$.

Carbon black (CB) Printex 90 (P90) particles were kindly provided by Degussa AG (Frankfurt am Main, Germany) and were reported to have a mean diameter of 14 nm. BET surface area was determined to be $323.20 \text{ m}^2/\text{g}$.

Crocidolite asbestos

Standard reference crocidolite asbestos (UICC) was obtained from SPI Supplies (Structure Probe Inc., West Chester, USA) and is characterized as described by Bowes and Farrow (1997). UICC crocidolite asbestos was reported to have a mean length of $\leq 5 \mu\text{m}$ and a mean width of $< 0.5 \mu\text{m}$ [Lang *et al.*, 2001]. BET surface area was reported to be $5.6 \text{ m}^2/\text{g}$ [Ono-Ogasawara and Kohyama, 1999].

Dispersion of test particles

Dispersion of carbon nanoparticles in cell culture medium

A stock concentration of 500 µg/ml SWCNT was prepared in the appropriate culture medium by vortexing the suspension three times for 5 seconds followed by sonication for 1 min in an ultrasonic bath (Sonorex RK52, Bandelin, Berlin, Germany). This procedure was repeated three times. Working concentrations of 50 µg/ml (25 µg/cm²) were prepared by 1:10 dilutions in culture medium.

Suspended in culture medium supplemented with FCS, HiPco SWCNT, arc discharge SWCNT and P90 particles were present as polydispersed agglomerates as determined by dynamic light scattering (Nano-ZS system, Malvern Instruments) and light microscopy (Zeiss confocal laser scanning fluorescence microscope LSM 510). Serum free culture medium led to more uniform P90 dispersion leading to agglomerates with an average diameter of 3640 nm. UV-vis and transmission electron microscopy (TEM) studies performed in our laboratory have indicated that SWCNT remain bundled at these concentrations after ultrasonic dispersion in culture medium [Casey *et al.*, 2007a; Casey *et al.*, 2007b; Herzog *et al.*, 2009].

Dispersion of carbon nanoparticles in DPPC

A DPPC (Sigma) solution of 2.5 mg/ml was prepared in PBS by sonication for 10 minutes at 37°C using an ultrasonic bath. A 500 µg/ml stock concentration of SWCNT was prepared in DPPC/PBS solution followed by vortexing three times for 5 seconds each and subsequently sonicating for 1 min (Sonorex RK52, Bandelin, Berlin, Germany). This procedure was repeated three times. From these stock concentrations, working concentrations of 50 µg/ml (25 µg/cm²) were prepared in A549 or NHBE medium.

Following DPPC dispersion, P90 samples showed improved dispersion in culture medium leading to smaller sized agglomerates of an average diameter of 677 nm as measured by DLS. It also improved SWCNT dispersion as recently described by Herzog *et al.* (2009), although no evidence of nanotube debundling was observed.

Dispersion of crocidolite asbestos

Stock concentrations of 500 µg/ml crocidolite asbestos $[(\text{Na}_2(\text{Fe}^{3+})_2(\text{Fe}^{2+})_3\text{Si}_8\text{O}_{22}(\text{OH})_2)]$ were prepared by diluting in the appropriate cell culture medium or DPPC/PBS. As asbestos was easily dispersible in aqueous solutions, no ultrasonication was required but short-term vortexing was used in order to disperse the samples. Working concentrations were prepared as described for carbon nanoparticles. Asbestos samples were present as singly dispersed fibers, independent of the dispersion medium used [Herzog *et al.*, 2009].

Carboxy-DCFDA assay

The carboxy-DCFDA assay was carried out as per manufacturer's instructions. Stock solutions of 10 mM carboxy-DCFDA (C-400, Molecular Probes) were prepared in DMSO and stored in the dark at -20°C. From this, a second stock solution of 1 mM was prepared in PBS immediately prior to use. A549 and NHBE cells were seeded at a density of 3×10^4 cell/well in 24 well plates and incubated at 37 °C for 24 hours for cells to adhere. Following 24 hours, culture medium was discarded and cells were washed with 1 ml PBS followed by addition of 500 µl of fresh culture medium. For 1 hour exposures, 5 µl of carboxy-DCFDA (1 mM) were added into each well and cells were incubated for 15 minutes at 37 °C. Afterwards, 50 µl of test particle solution (500 µg/ml) were added to

the cells (1:10 dilution). For 4 hour exposures, cells were first exposed to test particles by adding 50 μ l of test particle solution (500 μ g/ml) to each well. Following 3 hours incubation, 5 μ l of carboxy-DCFDA (1 mM) were added and cells were incubated for another 60 minutes. Hydrogen peroxide (H_2O_2) at a concentration of 500 μ M was used as positive control stimulus. Negative controls were exposed to the corresponding culture medium only. For DPPC exposures, culture medium containing 10% DPPC/PBS (0.25 mg/ml) was used as solvent control for DPPC exposures.

In parallel, cells were pre-treated with *N*-acetyl-l-cysteine (NAC) solution (25 mM) prepared in RPMI medium for 30 minutes before addition of carboxy-DCFDA or particle exposure.

At the end of the exposure period, cell supernatants were discarded, cells were washed with PBS and harvested using Trypsin/EDTA followed by suspension in PBS/FCS (10%). Cells were transferred into FACS tubes and 10000 events were immediately analyzed on the FITC-channel using flow cytometry (Becton Dickinson FACSCantoTM). Since previous experiments have shown that the addition of particles to A549 or NHBE cells did not cause significant cell death after 24 hours of incubation, no additional cell viability marker was included in the current study (Herzog *et al.*, 2009).

Statistical analysis

Experiments were conducted in duplicate, including two replicates each. The raw data were used (median fluorescence intensity) and the mean \pm standard deviation (SD) of duplicates or triplicates were calculated. Differences between samples and the control were evaluated using the statistical analysis package SPSS 15.0. Statistically significant

differences were set at $p \leq 0.05$. Normality of data was confirmed with Q-Q percentile plots and Kolmogorov-Smirnov tests. Equality of variances was evaluated using Levene tests. Factorial ANOVA were performed to determine whether overall treatment effects occurred within an experiment using a 95% confidence level. ANOVA was followed by Dunnett's multiple comparison tests were performed for normally distributed samples with homogeneous variances. Non-parametric tests, namely Kruskal-Wallis followed by Mann-Whitney-u-tests were applied to samples without normal distribution and/or inhomogeneous variances.

Results

The results of this study showed that in contrast to H₂O₂ treatment, exposure of NHBE cells to cell culture medium dispersed carbon nanoparticles or asbestos did not induce the formation of ROS (Figure 1). However, when the particles were dispersed in DPPC solution, a significant increase in ROS could be detected following HiPco SWCNT, arc discharge SWCNT, P90 and asbestos exposures whereby P90 was found to be the most active of the particles tested (Figure 1). Compared to cell responses to H₂O₂ (500 μM), ROS levels determined for agglomerated nanomaterials and asbestos are low to moderate. DPPC dispersion increased the ROS forming ability of particles compared to dispersion in DPPC free medium by a factor of 1.5, 2.2, 3.8 and 1.4 for HiPco SWCNT, ArcD SWCNT, P90 and asbestos, respectively (Figure 2).

As soon as NHBE cells were pre-treated with the antioxidant NAC, ROS formation was inhibited at all times (Figure 1). Neither particles nor H₂O₂ exposure was able to induce ROS in NAC pre-treated cells. In addition, NAC could decrease cellular ROS levels below that of untreated cells.

Incubation with NAC before particle exposure inhibited ROS formation also in A549 cells (data not shown). In the absence of NAC, HiPco and arc discharge SWCNT dispersed in FCS-free culture medium did result in slight intracellular ROS generation in this cell line with increases by factors of 1.9 and 1.1, respectively (Figure 3). When FCS was added as cell culture medium supplement, no ROS response could be detected in A549 cells for any of the particles tested (Figure 3).

Again, ROS formation was increased upon exposure to particle dispersions in DPPC (Figure 4). However, the difference was less pronounced than that demonstrated for NHBE cells. The highest increase could again be detected for P90 which resulted in an increase by 90% compared to culture medium (without FCS supplement) dispersed particles (Figure). The increases for HiPco and arc discharge SWCNT were not statistically significant.

Discussion

Oxidative stress has been identified as one of the best-developed paradigms to explain nanoparticle toxicity from a mechanistic point of view [Nel *et al.*, 2006]. It is also believed to be the main mechanism by which air pollution particles cause adverse effects in exposed tissues including epithelial cells and macrophages [Driscoll *et al.*, 1997; Li *et al.*, 2002]. Due to their large specific surface area, the reactivity and therefore the potential of nanoparticles to induce oxidative stress is particularly high [Donaldson *et al.*, 2006; Driscoll *et al.*, 1997; Li *et al.*, 2008; Nel *et al.*, 2006;; Oberdorster *et al.*, 2005a; Oberdorster *et al.*, 2005b].

In standard cell growth media, none of the particles tested were able to significantly increase ROS in NHBE cells following short-term exposure (Figure 1). In contrast, A549 cells significantly responded to HiPco SWCNT exposure, but not to any other particle (Figure 3). This might be explained by the use of different dispersion media. Where NHBE cells were exposed to particles suspended in specialized medium supplemented with a variety of growth factors and other additives, A549 cells were exposed to particles suspended in protein-free RPMI medium without the use of any supplements besides L-glutamine. The supplements present in NHBE medium might attenuate particle reactivity by coating their reactive surface area leading to decreased oxidative potential [Davoren *et al.*, 2007; Gao *et al.*, 2001]. The fact that ROS levels of A549 cells also returned to control levels as soon as particles were suspended in RPMI medium supplemented with FCS (Figure 4) also points to this conclusion. Therefore, the presence of additional protein seemed to have a protective effect, making particles un-reactive and supporting

the proposal that the protein corona formed around particles greatly influences particle toxicity [Lynch and Dawson, 2008].

Upon inhalation, particles may come into contact with lung surfactant which lines the alveoli and respiratory bronchioles in the deep lung [Schurch *et al.*, 1992]. This surfactant is synthesized by alveolar type II cells, such as A549 cells, in which it is stored in lamellar bodies [Dobbs *et al.*, 1987; Scarpelli, 1968]. Previous studies showed that exposure of A549 cells to HiPco SWCNT results in increased numbers of lamellar bodies, indicating an increase in surfactant production [Davoren *et al.*, 2007]. Therefore, the use of phospholipids as dispersing agent may be of great relevance for particle toxicity studies. DPPC, the major component of lung surfactant, dispersed in physiological saline, has been used as simple model of lung surfactant in particle toxicity studies [Foucaud *et al.*, 2007; Porter *et al.*, 2008; Sager *et al.*, 2007; Wallace *et al.*, 1992; Wallace *et al.*, 2007b]. In the case of silica toxicity, DPPC has been used as protective agent, inhibiting toxicity by particle coating [Hamilton *et al.*, 2008; Patzold *et al.*, 1993; Schimmelpfeng *et al.*, 1992; Wallace *et al.*, 1992]. However, it did not have a protective effect on chrysotile asbestos toxicity [Schimmelpfeng *et al.*, 1992]. In the presence of DPPC, the potential of carbon nanomaterials, namely SWCNT and carbon black, to induce intracellular ROS was significantly increased (Figures 5). The most pronounced increases could be seen for P90 exposure which resulted in increases of ROS by factors of 3.8 and 1.8 in NHBE and A549 cells, respectively. This agrees with studies by Foucaud *et al.* (2007) who also reported increased ROS production in monocytic cells following exposure to P90 suspended in DPPC compared to suspensions in culture

medium only. There was no statistically significant increase in asbestos toxicity. Overall, cells of primary origin, such as NHBE cells, are generally regarded as being more sensitive than carcinoma cell lines as represented by A549 cells [Kode *et al.*, 2006; Oostingh *et al.*, 2007]. This may explain why NHBE cells reacted differently to DPPC dispersion than A549 cells. The fact that A549 cells are surfactant producing alveolar type II cells may also play a role.

The increases in ROS in the presence of DPPC may be explained by improved dispersion of SWCNT and P90 particles. Interaction with DPPC may lead to hydrophilic coating of the particles, permitting dispersion in aqueous media, leading to greater surface areas available for reactions and therefore an increase in the registered toxic response as more cells would come into contact with particles. Improved dispersion due to lung surfactant coating was also reported to be the reason for increased genotoxicity of diesel exhaust particles (DEP) [Wallace *et al.*, 2007b]. As asbestos was well dispersed in culture medium only, the use of DPPC did not alter its reactivity. Improved dispersion of HiPco SWCNT upon DPPC dispersion could be measured using UV-vis spectroscopy as described by Herzog *et al.* (2009). Therefore, it is very likely that an increased number of cells will be exposed to SWCNT upon DPPC dispersion and nanotube-cell interactions may be increased. In addition, improved dispersion may also increase the risk of particle uptake by cells which would need to be investigated further.

Furthermore, as produced samples of SWCNT contain variable amounts of catalyst residues, most of which are transition metals such as iron, cobalt and nickel. The type of impurities present depends on the type of catalysts used during production, and thus a comparison of different nanotube type is merited. The importance of metals for eliciting

the release of pro-inflammatory cytokines from lung cells has been investigated in several studies [Carter *et al.*, 1997; Molinelli *et al.*, 2002]. Transition metals can generate ROS through Fenton and Haber-Weiss reactions [Carter *et al.*, 1997] and may also synergize with organic particulate matter (PM) components [Kodavanti *et al.*, 2002]. DPPC dispersion may lead to increased bioavailability of transition metal residues and other impurities present in SWCNT samples. As in previous studies, this study targets the assessment of the effects of as produced materials, as a mimic of occupational exposure. The highest transition metal content is for the HiPco nanotubes, but it is commonly accepted that the iron, in the form of a nanoparticle, is enclosed within the tip of the nanotube and thus is not bioavailable. Previous studies indicated that, when filtered to remove the carbon nanotubes, medium contained minimal amounts of trace iron. (Casey *et al.* (2008)). It is furthermore notable that, although HiPco SWCNT contain significantly larger amount of metal impurities, no correlation between the cellular response and metal content is observed.

The particulate matter content of chemicals has also been correlated with their capability to induce oxidative stress as seen in bronchial epithelial cells [Li *et al.*, 2003]. This organic particulate matter can synergize with transition metals in ROS generation [Kodavanti *et al.*, 2002]. Arc discharge produced SWCNT contain greater residues of amorphous carbon which may lead to increased toxicity compared to HiPco SWCNT in the presence of DPPC (Figure 2). In terms of factor increase, arc discharge nanotubes have a significantly larger effect than HiPco SWCNT on the NHBE cells indicating that the amorphous carbon residues contribute significantly in the better dispersed samples. However, this is not seen to be the case for the A549 cells.

In addition, DPPC can affect particle toxicity by altering their surface chemistry and ultimately changing their toxicity [Buford *et al.*, 2007; Hamilton *et al.*, 2008; Wallace *et al.*, 2007a; Wallace *et al.*, 2007b]. Any potential contribution to the observed effects can be established by investigations which are currently underway.

As a surfactant, DPPC forms liposomes in water solution and may form micellar structures through their interaction with the particles to aid dispersion. Therefore, it could be hypothesised that the change in packing of the DPPC molecules in the presence of the particle agglomerates may conceivably alter the chemical activity of the DPPC within the suspension which may render it more toxic.

Employing the same exposure conditions as used in the current investigation, previous studies indicated a suppression of inflammatory mediator release in A549 and NHBE cells following exposure to SWCNT over 48 hours. This was also seen in the presence of DPPC dispersion [Herzog *et al.*, 2009]. The fact that there is an increase in intracellular ROS but no apparent inflammatory response might indicate low levels of oxidative stress as represented by tier 1 of the hierarchical oxidative stress model [Halliwell and Gutteridge 1999; Nel *et al.*, 2006].

It has to be kept in mind that the carboxy-DCFDA assay is limited regarding the types of ROS it can detect, namely hydrogen peroxides, peroxy radicals and peroxy nitrite anions. Therefore, in order to obtain a full picture of oxidative stress within exposed cells, further assays would need to be carried out including further endpoints such as production of superoxide, lipid peroxidation products or catalase dependent hydrogen peroxides and cellular antioxidant states including GSH depletion.

Conclusion

This study illustrates that *in vitro* exposure of lung epithelial cells to carbon nanomaterials only results in moderate or low oxidative stress under the exposure conditions employed. However, ROS production as a result of carbon nanomaterial exposure was always higher compared to asbestos exposure. Cell responses seemed to strongly depend on the vehicle used for particle dispersion. Whereas interaction with DPPC appears to alter the particles making them more reactive, leading to higher ROS formation within the cells, the presence of FCS seemed to protect the cells from any oxidative insult. These findings may be of interest for occupational health risk assessment but also biomedical applications of the particles tested.

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References

Beck-Speier, I., Dayal, N., Karg, E., Maier, K. L., Schumann, G., Schulz, H., Semmler, M., Takenaka, S., Stettmaier, K., Bors, W., Ghio, A., Samet, J. M., and Heyder, J., 2005. Oxidative stress and lipid mediators induced in alveolar macrophages by ultrafine particles. *Free Radic Biol Med* **38**, 1080-1092.

Bianco, A., Kostarelos, K., and Prato, M., 2005. Applications of carbon nanotubes in drug delivery. *Current opinion in chemical biology* **9**, 674-679.

Briviba, K., Klotz, L. O., and Sies, H., 1997. Toxic and signaling effects of photochemically or chemically generated singlet oxygen in biological systems. *Biological chemistry* **378**, 1259-1265.

Buford, M. C., Hamilton, R. F., Jr., and Holian, A., 2007. A comparison of dispersing media for various engineered carbon nanoparticles. *Part Fibre Toxicol* **4**, 6.

Carter, J. D., Ghio, A. J., Samet, J. M., and Devlin, R. B., 1997. Cytokine production by human airway epithelial cells after exposure to an air pollution particle is metal-dependent. *Toxicol Appl Pharmacol* **146**, 180-188.

Casey, A., Davoren, M., Herzog, E., Lyng, F. M., Byrne, H. J., and Chambers, G., 2007a.
Probing the interaction of single walled carbon nanotubes within cell culture medium as a precursor to toxicity testing. *Carbon* **45**, 34-40.

Casey, A., Herzog, E., Davoren, M., Lyng, F. M., Byrne, H. J., and Chambers, G., 2007b.
Spectroscopic analysis confirms the interactions between single walled carbon nanotubes and various dyes commonly used to assess cytotoxicity. *Carbon* **1**.

“Single walled carbon nanotubes induce indirect cytotoxicity by medium depletion in A549 lung cells.”
A. Casey, E. Herzog, F.M. Lyng, H.J. Byrne, G. Chambers and M. Davoren, *Toxicology Letters*, 179, 78-84 (2008)

Davoren, M., Herzog, E., Casey, A., Cottineau, B., Chambers, G., Byrne, H. J., and Lyng, F. M., 2007. In vitro toxicity evaluation of single walled carbon nanotubes on human A549 lung cells. *Toxicol In Vitro* **21**, 438-448.

Dobbs, L. G., Wright, J. R., Hawgood, S., Gonzalez, R., Venstrom, K., and Nellenbogen, J., 1987. Pulmonary surfactant and its components inhibit secretion of phosphatidylcholine from cultured rat alveolar type II cells. *Proc Natl Acad Sci U S A* **84**, 1010-1014.

Donaldson, K., Aitken, R., Tran, L., Stone, V., Duffin, R., Forrest, G., and Alexander, A., 2006. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. *Toxicol Sci* **92**, 5-22.

- Driscoll, K. E., Carter, J. M., Hassenbein, D. G., and Howard, B., 1997. Cytokines and particle-induced inflammatory cell recruitment. *Environmental health perspectives* **105 Suppl 5**, 1159-1164.
- Foucaud, L., Wilson, M. R., Brown, D. M., and Stone, V., 2007. Measurement of reactive species production by nanoparticles prepared in biologically relevant media. *Toxicol Lett* **174**, 1-9.
- Gao, N., Keane, M. J., Ong, T., Ye, J., Miller, W. E., and Wallace, W. E., 2001. Effects of phospholipid surfactant on apoptosis induction by respirable quartz and kaolin in NR8383 rat pulmonary macrophages. *Toxicol Appl Pharmacol* **175**, 217-225.
- Halliwell, B., Gutteridge, J. M. C., 1999. Free radicals in biology and medicine. 3rd ed., Oxford Univ Press, Oxford.
- Hamilton, R. F., Jr., Thakur, S. A., and Holian, A., 2008. Silica binding and toxicity in alveolar macrophages. *Free Radic Biol Med* **44**, 1246-1258.
- Hedderman T. G., 2006. PhD Thesis, 91-103. Dublin.
- Herzog, E., Byrne, H. J., Casey, A., Davoren, M., Lenz, A. G., Maier, K. L., Duschl, A., Oostingh, G. J., 2008. SWCNT suppress inflammatory mediator responses in human lung epithelium in vitro. *Toxicol Appl Pharm*, **234**, 378-390 (2009).

- Huczko, A., Lange, H., Calko, E., Grubek-Jaworska, H., Droszez, P., 2001. Physiological testing of carbon nanotubes: are they asbestos-like? *Fullerene Sci and Techn* **9**, 251-254.
- Kodavanti, U. P., Schladweiler, M. C., Ledbetter, A. D., Hauser, R., Christiani, D. C., Samet, J. M., McGee, J., Richards, J. H., and Costa, D. L., 2002. Pulmonary and systemic effects of zinc-containing emission particles in three rat strains: multiple exposure scenarios. *Toxicol Sci* **70**, 73-85.
- Kode, A., Yang, S. R., and Rahman, I., 2006. Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. *Respiratory research* **7**, 132.
- Lacerda, L., Bianco, A., Prato, M., and Kostarelos, K., 2006. Carbon nanotubes as nanomedicines: from toxicology to pharmacology. *Advanced drug delivery reviews* **58**, 1460-1470.
- LaDou, J., 2004. The asbestos cancer epidemic. *Environmental health perspectives* **112**, 285-290.

- Lang, D. S., Schocker, H., and Hockertz, S., 2001. Effects of crocidolite asbestos on human bronchoepithelial-dependent fibroblast stimulation in coculture: the role of IL-6 and GM-CSF. *Toxicology* **159**, 81-98.
- Li, N., Sioutas, C., Cho, A., Schmitz, D., Misra, C., Sempf, J., Wang, M., Oberley, T., Froines, J., and Nel, A., 2003. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. *Environmental health perspectives* **111**, 455-460.
- Li, N., Wang, M., Oberley, T. D., Sempf, J. M., and Nel, A. E., 2002. Comparison of the pro-oxidative and proinflammatory effects of organic diesel exhaust particle chemicals in bronchial epithelial cells and macrophages. *J Immunol* **169**, 4531-4541.
- Li, N., Xia, T., and Nel, A. E., 2008. The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. *Free Radic Biol Med* **44**, 1689-1699.
- Lynch, I., Dawson, K. A. 2008. Protein-nanoparticle interactions. *Nanotoday* **3**, 40-47.
- Maynard, A., Baron, P., Foley, M., Shvedova, A., Kisin, E., and Castranova, V., 2004a. Exposure to carbon nanotube material: aerosol release during the handling of

- unrefined single-walled carbon nanotube material. *J Toxicol Environ Health A* **67**, 87-107.
- Maynard, A. D., Baron, P. A., Foley, M., Shvedova, A. A., Kisin, E. R., and Castranova, V., 2004b. Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-walled carbon nanotube material. *J Toxicol Environ Health A* **67**, 87-107.
- Molinelli, A. R., Madden, M. C., McGee, J. K., Stonehuerner, J. G., and Ghio, A. J., 2002. Effect of metal removal on the toxicity of airborne particulate matter from the Utah Valley. *Inhalation toxicology* **14**, 1069-1086.
- Nel, A., Xia, T., Mädler, L., Ning, L., 2006. Toxic potential of materials at the nanolevel. *Science* **311**, 622-627.
- Oberdorster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., Carter, J., Karn, B., Kreyling, W., Lai, D., Olin, S., Monteiro-Riviere, N., Warheit, D., and Yang, H., 2005a. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. *Part Fibre Toxicol* **2**, 8.

Oberdorster, G., Oberdorster, E., and Oberdorster, J., 2005b. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environmental health perspectives* **113**, 823-839.

Ono-Ogasawara, M., Kohyama, N., 1999. Evaluation of surface roughness of fibrous minerals by comparison of BET surface area and calculated one. *Ann Occup Hyg* **43**, 505-511.

Oostingh, G. J., Schlickum, S., Friedl, P., and Schon, M. P., 2007. Impaired induction of adhesion molecule expression in immortalized endothelial cells leads to functional defects in dynamic interactions with lymphocytes. *The Journal of investigative dermatology* **127**, 2253-2258.

Patzold, S., Schmidt, A., and Seidel, A., 1993. Loss of cathepsin B activity in alveolar macrophages after in vitro quartz phagocytosis. *Journal of toxicology and environmental health* **40**, 547-554.

Porter, D., Sriram, K., Wolfarth, M., Jefferson, A., Schwegler-Berry, D., Andrew, M. E., and Castranova, V., 2008. A biocompatible medium for nanoparticle dispersion. *Nanotoxicology* **2**, 144-154.

Royal Society and Royal Academy of Engineering, 2004. Nanoscience and nanotechnologies: opportunities and uncertainties. Royal Society Publications, London, available online www.royalsoc.ac.uk/policy.

Sager, T. M., Porter, D. W., Robinson, V. A., Lindsley, W. G., Schwegler-Berry, D. E., Castranova, V., 2007. Improved method to disperse nanoparticles for in vitro and in vivo investigation of toxicity. *Nanotoxicology* **1**, 118-129.

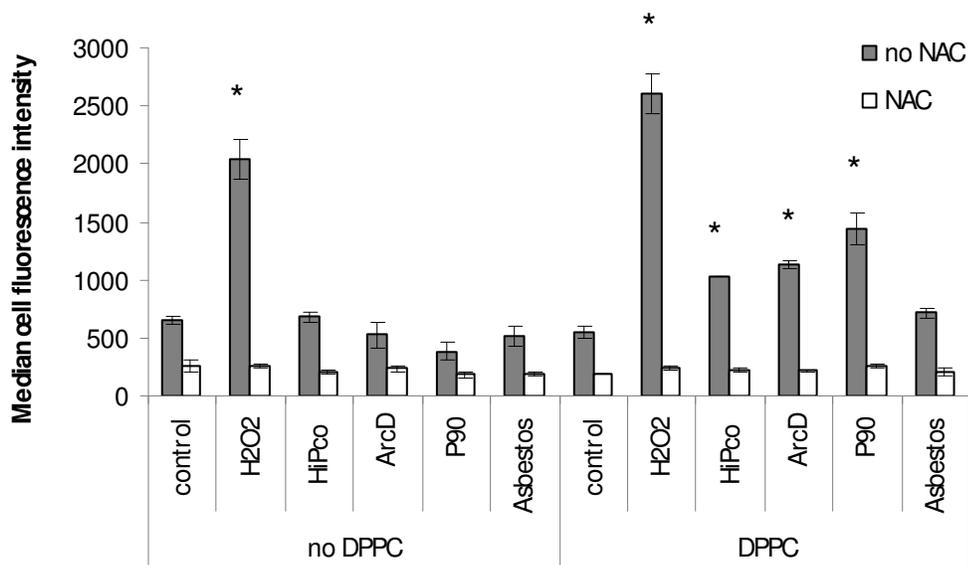
Sayes, C. M., Gobin, A. M., Ausman, K. D., Mendez, J., West, J. L., and Colvin, V. L., 2005. Nano-C60 cytotoxicity is due to lipid peroxidation. *Biomaterials* **26**, 7587-7595.

Scarpelli, E. M., 1968. Lung surfactant: dynamic properties, metabolic pathways, and possible significance in the pathogenesis of the respiratory distress syndrome. *Bulletin of the New York Academy of Medicine* **44**, 431-445.

Schimmelpfeng, J., Drosselmeyer, E., Hofheinz, V., and Seidel, A., 1992. Influence of surfactant components and exposure geometry on the effects of quartz and asbestos on alveolar macrophages. *Environmental health perspectives* **97**, 225-231.

- Schurch, S., Bachofen, H., Goerke, J., and Green, F., 1992. Surface properties of rat pulmonary surfactant studied with the captive bubble method: adsorption, hysteresis, stability. *Biochim Biophys Acta* **1103**, 127-136.
- Shukla, A., Gulumian, M., Hei, T. K., Kamp, D., Rahman, Q., and Mossman, B. T., 2003. Multiple roles of oxidants in the pathogenesis of asbestos-induced diseases. *Free Radic Biol Med* **34**, 1117-1129.
- Shvedova, A. A., Castranova, V., Kisin, E. R., Schwegler-Berry, D., Murray, A. R., Gandelsman, V. Z., Maynard, A., and Baron, P., 2003. Exposure to carbon nanotube material: assessment of nanotube cytotoxicity using human keratinocyte cells. *J Toxicol Environ Health A* **66**, 1909-1926.
- Smart, S. K., Cassady, A. I., Lu, G. Q., Martin, D. J., 2006. The biocompatibility of carbon nanotubes. *Carbon* **44**, 1034-1047.
- Vallyathan, V., and Shi, X., 1997. The role of oxygen free radicals in occupational and environmental lung diseases. *Environmental health perspectives* **105 Suppl 1**, 165-177.
- Wallace, W. E., Keane, M. J., Gautam, M., Shi, X. C., Murray, D., Ong, T., 2007a. Dispersion of nanoparticles in pulmonary surfactants for in vitro toxicity studies: Lessons from ultrafine diesel exhaust particles and fine mineral dusts. In:

- Monteiro-Riviere, N.A. and Tran, L. (Eds.), *Nanotoxicology: Characterization, Dosing and Health Effects*, Informa Healthcare, New York, London, pp. 153-172.
- Wallace, W., Keane, M., Murray, D., Chisholm, W., Maynard, A., and Ong, T.-M., 2007b. Phospholipid lung surfactant and nanoparticle surface toxicity: Lessons from diesel soots and silicate dusts. In *Nanotechnology and Occupational Health*, pp. 23-38.
- Wallace, W. E., Keane, M. J., Mike, P. S., Hill, C. A., Vallyathan, V., and Regad, E. D., 1992. Contrasting respirable quartz and kaolin retention of lecithin surfactant and expression of membranolytic activity following phospholipase A2 digestion. *Journal of toxicology and environmental health* **37**, 391-409.
- Xia, T., Korge, P., Weiss, J. N., Li, N., Venkatesen, M. I., Sioutas, C., and Nel, A., 2004. Quinones and aromatic chemical compounds in particulate matter induce mitochondrial dysfunction: implications for ultrafine particle toxicity. *Environmental health perspectives* **112**, 1347-1358.
- Xia, T., Kovoichich, M., Brant, J., Hotze, M., Sempf, J., Oberley, T., Sioutas, C., Yeh, J. I., Wiesner, M. R., and Nel, A. E., 2006. Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. *Nano Lett* **6**, 1794-1807.



Figures

Figure 1: ROS formation in NHBE cells following exposure to 50 µg/ml carbon nanoparticles and asbestos dispersed in NHBE medium with and without NAC pre-treatment in the presence and absence of DPPC dispersion. Data are expressed as median cell fluorescence intensity ± SD. *Denotes a significant difference from the control ($P \leq 0.05$). NAC pre-treatment was significant in all cases ($P < 0.05$), therefore no asterisks are shown.

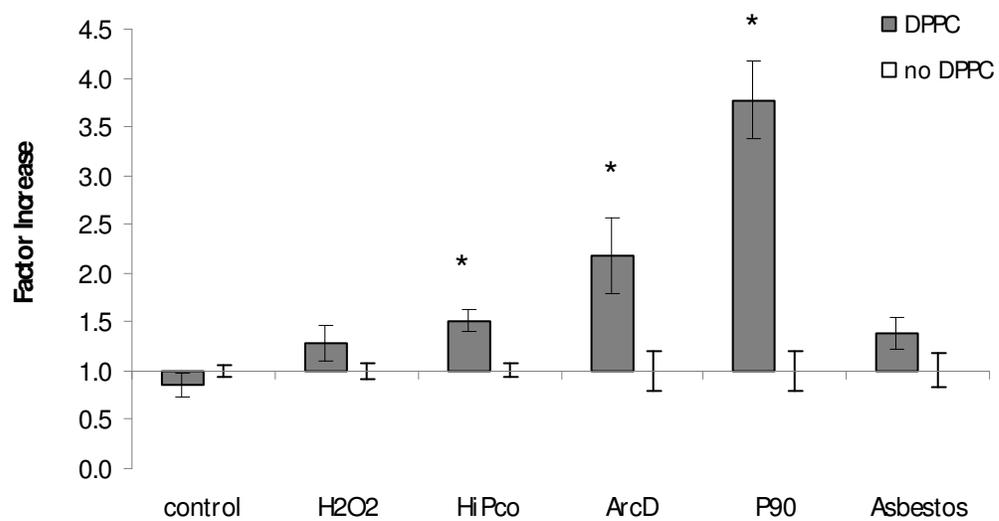


Figure 2: Comparison of ROS formation in NHBE cells in the presence (grey) and absence (white) of DPPC dispersion. Data are expressed as factor increase over control \pm SD. *Denotes a significant effect of DPPC dispersion ($P \leq 0.05$).

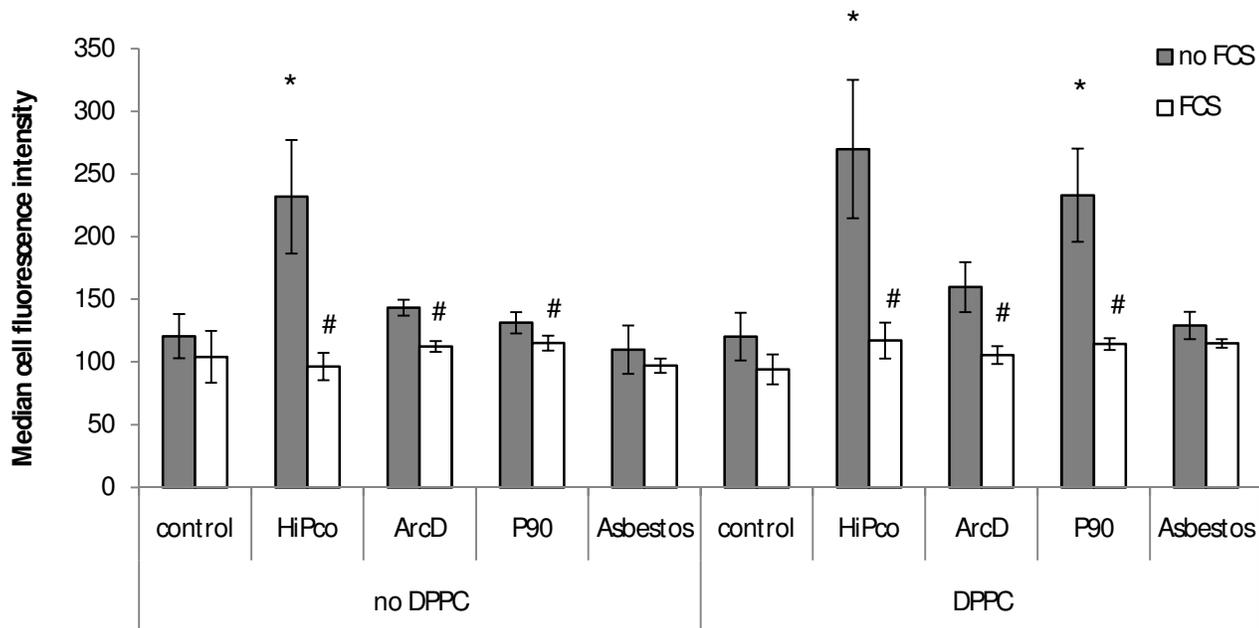


Figure 3: ROS formation in A549 cells following exposure to 50 µg/ml carbon nanoparticles and asbestos dispersed in RPMI medium with and without FCS supplement in the presence and absence of DPPC. Data are expressed as median cell fluorescence intensity ± SD. *Denotes a significant difference from the control ($P \leq 0.05$). #Denotes a significant effect of FCS addition.

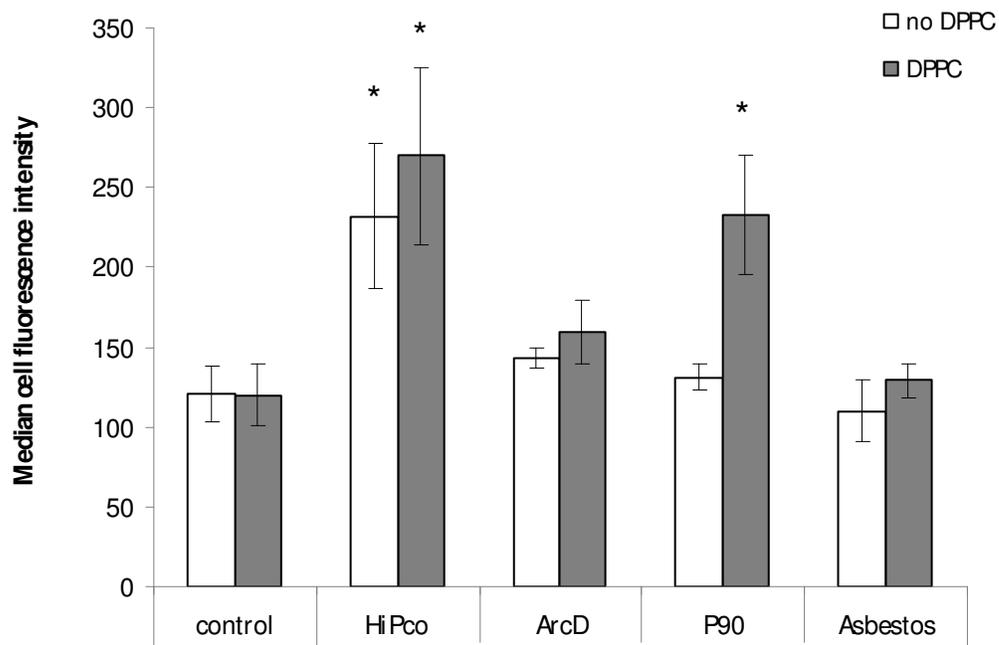


Figure 4: ROS formation in A549 cells following exposure to 50 µg/ml carbon nanoparticles dispersed in RPMI medium or DPPC. Data are expressed as median cell fluorescence intensity ± SD.

***Denotes a significant effect of DPPC dispersion ($P \leq 0.05$).**