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Phospholipid lung surfactant and nanoparticle surface toxicity: Lessons from diesel soots and silicate dusts

William E. Wallace[1,2,*], Michael J. Keane[1], David K. Murray[1], William P. Chisholm[1], Andrew D. Maynard[3] and Tong-man Ong[1]
[1]*US National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV, 26505, USA;* [2]*College of Engineering and Mineral Resources, West Virginia University, Morgantown, WV, 26506, USA;* [3]*Woodrow Wilson International Center for Scholars, Woodrow Wilson Plaza, 1300 Pennsylvania Avenue, N.W., Washington, DC, 2004-3027, USA; *Author for correspondence (Tel.: +304-285-6096; E-mail: wwallace@cdc.gov)*

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Abstract

Because of their small size, the specific surface areas of nanoparticulate materials (NP), described as particles having at least one dimension smaller than 100 nm, can be large compared with micrometer-sized respirable particles. This high specific surface area or nanostructural surface properties may affect NP toxicity in comparison with micrometer-sized respirable particles of the same overall composition. Respirable particles depositing on the deep lung surfaces of the respiratory bronchioles or alveoli will contact pulmonary surfactants in the surface hypophase. Diesel exhaust ultrafine particles and respirable silicate micrometer-sized insoluble particles can adsorb components of that surfactant onto the particle surfaces, conditioning the particles surfaces and affecting their *in vitro* expression of cytotoxicity or genotoxicity. Those effects can be particle surface composition-specific. Effects of particle surface conditioning by a primary component of phospholipid pulmonary surfactant, diacyl phosphatidyl choline, are reviewed for *in vitro* expression of genotoxicity by diesel exhaust particles and of cytotoxicity by respirable quartz and aluminosilicate kaolin clay particles. Those effects suggest methods and cautions for assaying and interpreting NP properties and biological activities.

Concerns for health hazard from nanoparticulate exposures

Research has demonstrated the importance of parameters such as size and number in determining the toxicity of insoluble particles with nanometer dimensions, or nanoscale structures (Oberdörster et al., 1995, 2004; Driscoll, 1996; Donaldson et al., 2000; Oberdörster 2000; Tran et al., 2000). Nanostructured materials including nanoparticles (NP) are defined as having at least one dimension smaller than 100 nm (Maynard & Kuempel, 2005). There also is concern for surface property effects on NP-induced toxicity or disease risk. This is due to the large specific surface area, i.e., surface area per unit mass, of NP associated with their small size, and because surface area and surface properties can strongly affect the toxicity or disease risk

associated with respirable micrometer-sized particles. Therefore, while such possible effects are under investigation, NP as administered for cellular or animal model bioassay ideally should not be altered in size, morphology, aggregation and surface properties from their condition upon deposition in the lung after workplace or environmental inhalation exposure. As part of this, a critical concern is the conditioning of NP that will occur upon the initial deposition of particles upon the aqueous hypophase environmental interface of the lung, e.g., by adsorption of and dispersion in biomolecular components of lung surfactant or serum.

NP may differ on a mass basis from larger particles of the same composition for expression of toxicity and for biological transport and bioavailability because of higher specific surface area of NP. Materials deemed low in toxicity as larger particles may exhibit toxic effects as NP. Greater toxicity is reported for ultrafine carbon black, TiO_2 and latex particles compared to larger low-toxicity low-solubility particles of the same material (Donaldson et al., 2000); a tenfold increase in inflammation observed for the same mass of ultrafine versus fine particles was attributed to increased oxidative activities of the ultrafine particles. A set of dusts of low toxicity when in the micrometer size range including TiO_2, talc, carbon black, and photocopier toner, and particles with some toxicity including coal mine dust and diesel exhaust particulate material (DPM), were found to have comparable toxicity on a surface area basis for lung tumor induction in a rat model; and that toxicity increased strongly with increase in dose measured as surface area (Maynard & Kuempel, 2005). However, in some cases there is a strong mineral specific component of toxicity not resolved by surface area normalization of dose; for example, fine-sized crystalline silica, e.g., quartz dust, is much more active than TiO_2 for pulmonary inflammation in an animal model (Oberdörster et al., 1994). Differing degrees of inflammation and lung injury upon ultrafine NiO, Co_3O_4, TiO_2 and carbon black instillation in rat lung have been reported (Dick et al., 2003). Degree of lung injury was found to correlate to the particle's ability to generate surface free radicals and to cause oxidant damage. Surface area, chemical composition and surface reactivity were all deemed important factors in particle toxicity. Exacerbated pulmonary inflammation may be a means by which airborne pollutant matter (PM) exerts its toxicity. (Tao et al., 2003). The smallest PM, below 2.5 μm, was most consistently associated with toxicity; the toxicity was attributed to oxidative stress caused by reactive oxygen species associated with metal, semi-quinone, lipopolysaccharide, or hydrocarbon constituents of ultrafine particles.

NP also may be able to cross the cell membrane and enter the bloodstream from the lungs (Ferin & Oberdörster, 1992; Oberdörster et al., 1992; Geiser et al., 2005). This general cell-penetrating ability is known, and even exploited, in the field of in vivo imaging. NP with special fluorescent, magnetic or optical properties such as "quantum dots" and magnetic resonance imaging contrast agents are functionalized with biocompatible coatings such as peptides, polysaccharides or other polymers and then directed within cells to permit selective signaling from specific cell components (Michalet et al., 2005; Sadeghiani et al., 2005). This ability to cross cell membranes has been pursued to provide functionalizing agents to transport peptides and DNA fragments into cells, e.g., through the endothelial tight-junction blood–brain barrier (Pantarotto et al., 2003, 2004a, b; Lu et al., 2005; Zhi et al., 2005). Such an uncommon effect is reported in studies of a variety of inorganic NP (Peters, et al., 2004). Cytotoxicity is a concern in new applications of NP, and safe exposure levels must be determined before these agents can be used in medical procedures. The majority of NP surveyed (TiO_2, SiO_2, and Co) were internalized into human epithelial cells, though most did not show cytotoxic effects. A pro-inflammatory stimulation and impairment of proliferative activity was observed for nano-Co and nano-SiO_2 particles, which was speculated to lead to a chronic inflammatory response and subsequent development of granulomas.

Carbonaceous materials represent a major class of NP, and a wide range of toxicity may result from variations in their shape, size, and complex chemical composition. The spherical nanoparticulate soot fullerene (C_{60}) was intentionally produced in 1985 by laser ablation of graphite targets. Limited toxicity studies of fullerene indicate this material was toxic to fish in aqueous systems, where fullerenes were found to pass the blood–brain barrier and cause brain damage (Oberdorster, 2004). The discovery of fullerenes has led to

new categories of NP carbonaceous products with a variety of useful shapes, including multi-walled and single-walled carbon nanotubes (CNT). CNT may exhibit significant cell toxicity on the basis of the combined effects of quantum physical effects such as cell wall penetration and the toxicity observed for similar carbonaceous bulk materials. Other studies show evidence of toxic behavior, though the mechanisms involved are not described. CNT may inhibit HEK293 cell growth by inducing cell apoptosis and decreasing cellular adhesion ability (Cui et al., 2005). CNT adverse affects are reported for immortalized human epidermal keratinocytes (Shvedova et al., 2003). Oxidative stress and cellular toxicity was indicated by the formation of free radicals, the accumulation of peroxidative products, antioxidant depletion and loss of cell viability. Recent *in vitro* studies of NP have reported cytotoxic activities for C_{60} and multi-walled CNT, and also for Ag, TiO_2, Fe_2O_3, Al_2O_3, ZrO_2, Si_3N_4, carbon black, and MnO_2 NP (Bottini et al., 2005; Gurr et al., 2005; Hussain et al., 2005; Sayes et al., 2005; Soto et al., 2005).

In an animal model study, three single-walled carbon nanotube materials (SWCNT) containing different amounts of residual metals were intratracheally instilled into mice at three doses and histopathology performed at 7 and 90 d (Lam et al., 2004). All the SWCNT induced dose-dependent epithelioid granulomas and in some cases interstitial inflammation at 7 d, with progression at 90 d, demonstrating greater toxicity on a mass basis than a carbon black negative control and a quartz dust positive control. Somewhat different response was observed in a study using SWCNT instillation in a rat model at two doses, with bronchiolar lavage biomarker assay and tissue histopathology at 24 h, 1 week, 1 month, and 3 months (Warheit et al., 2004). There, SWCNT exposures resulted in transient inflammatory response and injury, with non-dose-dependent multi-focal granulomas that did not progress after 1 month, and lack of toxicity indicated by lung lavage and cell proliferation measures. To address the somewhat disparate results of these two studies, a complete evaluation was performed of the dose dependence and time course of pulmonary response of mice to single pharyngeal aspiration exposure of purified SWCNT at doses bracketing the equivalent of 20 workdays of exposure at the OSHA Permissible Exposure Limit (PEL) for graphite particles (Shvedova et al., 2005). The study found acute inflammation and granulomatous response associated with dense SWCNT aggregates and, interestingly, early onset of progressive diffuse interstitial fibrosis and alveolar wall thickening associated with dispersed SWCNT distant from the aggregates. Protein, lactate dehydrogenase (LDH), and oxidative biomarkers were increased in bronchoalveolar lavage. Equal mass doses of ultrafine carbon black or fine crystalline silica dust caused weaker inflammation and damage, and no granulomas or cell wall thickening. *In vitro* macrophage exposures in the same study found TGF-beta 1 production and a weaker TNF-alpha and IL-1 beta production was stimulated by the SWCNT, but no stimulation of superoxide, NO, active engulfment, or apoptosis.

Genotoxicity of ultrafine DPM

DPM are anthropogenic, inadvertently generated organic NP materials. The National Institute for Occupational Safety and Health (NIOSH, 1988), the International Agency for Research on Cancer (IARC, 1989) and the US Environmental Protection Agency (USEPA, 2002) have declared diesel exhaust a potential or probable human carcinogen. DPM and carbonaceous materials from combustion processes are generally a complex mix of aromatic carbon graphitic sheets as a core, with reactive oxygen, nitrogen or sulfur heteroatomic functional groups, plus metals and organic species entrained during the synthesis or combustion process. Polycyclic aromatic hydrocarbon (PAH) compounds that are known carcinogens are contained in some of these products. Genotoxicity of carbonaceous material in NP may be attributed to PAH carcinogenicity, oxidation from reactive oxygen species (ROS) activity, nitrate generation, or transition metal chemistry. In vivo animal studies of DPM have confirmed lung tumors in the rat from long-term inhalation exposures (Heinrich et al., 1986; Mauderly et al., 1987). However, the genotoxic role of DPM in inhalation tumorogenicity studies has been questioned after comparable tests of non-genotoxic carbon black resulted in tumorogenesis in the same animal models (Heinrich et al., 1994; Nikula et al., 1995). DPM are insoluble in water, and typically are prepared for

chemical study or *in vitro* bioassay by solution in organic solvents and fractionation by chromatographic techniques. DPM typically contain a number of proven toxic species; polar hetero- and polycyclic aromatics, radical species, entrained metal species, and entrained organic solvent species, with the composition varying with engine performance characteristics, fuel types, lube oil types, and extraction solvents (Morimoto, 1986). These species have been fractionated and measured in DPM using bulk mass, particle size, as well as advanced chromatographic and mass spectroscopic techniques.

In vitro genotoxic activities elicited by organic solvent extracts, e.g., acetone or dichloromethane solvent extracts of some filter-collected DPM, are well-reported (IARC, 1989). The use of extraction solvents has led to discrepancies in assigning genotoxic effects to chemical characteristics (Hayakawa et al., 1997; Soontjens et al., 1997; Saxena et al., 2003; Siegel et al., 2004). Apparent false negatives or positives have been attributed to solvent/adsorbed organic dispersion, solvency or matrix effects. However, the *in vitro* mutagenicity of solvent extract of DPM can vary systematically with operating conditions for a given engine, e.g., with engine speed and torque (McMillian et al., 2002).

The lack of water-solubility of the polycyclic organics from DPM raised the question of their biological availability for genotoxic activity under conditions of particle deposition in the lung. It was recognized that the conventional procedure of testing organic solvent extracts of DPM did not necessarily provide a physiologically reasonable model of genotoxicant biological availability as manifest by intact particles deposited in the lung. Therefore, research examined as a medium for *in vitro* cellular challenge, the extract of DPM by primary components of the surfactant hypophase layer that coats the deep lung respiratory bronchioles and alveoli, frequently using diacyl phosphatidyl cholines dispersed into physiological saline.

Surrogate lung surfactant

The environmental interface of the deep lung respiratory bronchioles and alveoli for initial contact with inhaled particles is the surfactant-coated and laden hypophase (reviewed in Bourbon (1991)). Lipids and lipoprotein surfactants are synthesized and secreted onto the wet surface of the deep lung by alveolar type II cells. Additional biochemical ingredients, e.g., components of mucus, are found in the hypophase of the upper airways as part of the mucociliary system for lung clearance. Phospholipids are a major component of lung surfactant. By themselves they can reproduce physiologically important surface-tension properties of the pulmonary alveolar hypophase surface (Scarpelli, 1968). Research modeling lung surface tension phenomena often has used a major phospholipid component of pulmonary surfactant, dipalmitoyl phosphatidyl choline (DPPC) or some other diacyl phosphatidylcholines, dispersed into physiologic saline, as a simple model of lung surfactant. This surfactant has been used to model the possibility of lung surfactant extraction of genotoxicants from DPM. Experiments incorporated DPPC dispersed into physiological saline as a solvent to attempt to extract filter-collected DPM. However, this produced an extract with little or no genotoxic activity (Brooks et al., 1981; King et al., 1981; McClellan et al., 1982; Wallace et al., 1987), even when the organic solvent extract of a parallel DPM sample expressed significant activity. Instead, it was found that some DPM can express genotoxic activity, e.g., DNA or chromosomal damage *in vitro*, as a dispersion in this surfactant. DPM was tested as a direct mixture into such surfactant, without subsequent filtering; that is, the DPM was tested as a non-dissolved but surfactant-dispersed particulate in surfactant mixture (Wallace et al., 1987, 1990a; Keane et al., 1991; Gu et al., 1992, 1994, 2005). DPM genotoxic activities are expressed when the DPM is dispersed into DPPC surfactant; and those activities are associated with the non-dissolved particulate phase material. DPPC dispersion does not extract genotoxicants from the DPM particles; rather, the phospholipid coats and "solubilizes" (not "dissolves") the DPM, providing a hydrophilic coating and permitting the dispersion of the surfactant-coated DPM as particles in aqueous media.

To first order, this dispersion of collected DPM into the principal component of lung surfactant should adulterate the collected DPM approximately to the degree that the particles would be conditioned upon deposition in the deep lung, causing particle agglomeration or disassociation

and particle surface conditioning to approximately the same degree as would occur in the lung alveolar hypophase. That is, assaying DPM dispersed (mixed) into lavaged or synthetic models of lung alveolar hypophase surfactant avoids to first order the non-physiologic dissolution of particles and associated destruction of particulate properties, as would occur in organic solvent extraction of collected DPM. This provides a physiologically reasonable representation *in vitro* of toxicant bioavailability for particles depositing in the lung.

After filter-collected DPM is mixed into a dispersion of DPPC surfactant in saline, the then wettable-surfaced nano-particles can challenge cells effectively to express genotoxic activities for mammalian cell DNA and clastogenic damage, as well as for bacterial cell mutagenicity. This provides a physiologically reasonable method for handling and delivery of DPM for toxicological assays which might be applicable to other insoluble hydrophobic NP materials. And the phenomenon of surfactant-dispersed DPM expression of genotoxic activities suggests a first mechanistic step by which cells can be effectively exposed to insoluble NP genotoxicants following inhalation and deposition on the deep lung pulmonary respiratory bronchioles or alveoli.

The basis for the surfactant activity of DPPC is that the molecular structure contains a hydrophilic end and a hydrophobic end (Figure 1). The former consists of a trimethyl ammonium·cationic group bound through a two-carbon chain to an acidic phosphate, forming a zwitterionic dipole and providing a hydrophilic moiety as one end of the molecule. The phosphate is esterified to the first carbon of a glycerol which is esterified at the other two carbons to two long chain fatty acid residues, palmitate in the case of DPPC. These provide two hydrophobic, lipophilic long tails to the molecule. When dispersed into aqueous media, the phospholipid molecules aggregate into multi-molecular structures such that the hydropilic zwitterionic head groups of the molecules are oriented to face into the surrounding water molecules, while the hydrophobic fatty acid tails cluster amongst themselves, minimizing contact with water or the hydrophilic heads of other phospholipids. This gives rise to spherical or lamellar structures made up of a bilayer of surfactant molecules. The zwitterionic head groups are on the outer surfaces of the bilayer, with the lipid tails "sandwiched" between. This structure is the general basis for the bilayer phospholipid membrane underlying cell membrane structure.

DPPC can be dispersed into these liposomal and lamellar bilayer structures by ultrasonication into saline, forming a pale milky stable dispersion. When collected DPM is mixed into this DPPC dispersion the DPM soot particle agglomerates are observed to disperse. The DPM is "solubilized", that is, dispersed as small particles rather than dissolved into the DPPC dispersion. In this DPM-in-DPPC dispersion, the long chain lipophilic/hydrophobic tails of the DPPC molecule associate with the organic DPM particle surfaces, while the zwitterionic hydrophilic trimethyl ammonium and phosphate head of the molecule orients outward to face the surrounding aqueous medium. A simplified picture is that of a DPM particle as a tar "pin-cushion" covered by DPPC soap molecules with their tails stuck as the shaft of pins to the pin-cushion DPM particle and their heads outward, providing a hydrophilic outer coating, in-turn permit-

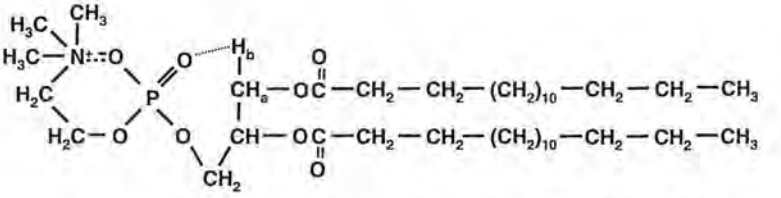

Figure 1. DPPC surfactant structure: Palmitate residues associate with DPM hydrocarbon; The zwitterionic trimethyl ammonium – acidic phosphate end of the molecule is hydrophilic, the two fatty acid tails esterified to the glycerol are hydrophobic and lipophilic, leading the molecules to aggregate in structures in aqueous media with the hydrophilic moieties oriented toward the water. The hydrophobic end of the molecule will associate with those tails of other lipid molecules or with the surface of hydrophobic particles, e.g., diesel exhaust particles, providing a "wettable" surface on a surfactant-coated DPM particle.

ting the structure to act as a water-wet but non-dissolved NP which disperses in water.

In vitro genotoxicity assays of DPM dispersed in surfactants

Comparisons of *in vitro* genotoxicites have been made between surfactant-dispersed and solvent-extracted DPM (Wallace et al., 1987, 1990a; Keane et al., 1991; Gu et al., 1992, 1994, 2005).

The DPM tested was filter-collected and graciously supplied by the Lovelace-Inhalation Toxicology Research Institute from a 1980 GM 5.7 liter V8 engine run on the Federal Test Procedure Urban Driving Cycle. For organic solvent extracted sample, DPM was dissolved into dichloromethane (DCM) and evaporatively exchanged into dimethylsulfoxide (DMSO) at 1 mg DPM/ml DMSO. For the surfactant dispersion sample, the surfactant was prepared by ultrasonically dispersing DPPC into physiological saline (PSS) at 2.5 mg DPPC/1 ml PSS; then DPM was mixed into that dispersion at 1 mg DPM/ 2.5 mg DPPC / 1 ml PSS. The tested materials were (a) the total preparations, in DMSO and in DPPC/PSS; (b) supernatants from centrifugation and filtration of the total preparations; and/or (c) sediments from centrifugation of the total preparations, i.e., the non-dissolved particulate phase material.

The Ames *Salmonella typhimurium* TA98 was used for the mutagenicity assay (Ames et al., 1975). As shown in Figure 2 (Keane et al., 1991), the total dispersion samples, both solvent and surfactant total preparations, were comparably mutagenic. The supernatant fraction (extracted material) of the solvent preparation was positive and of the surfactant preparation supernatant was negative; i.e., no active mutagenic material extracted from DPM by surfactant. The sediment fraction (non-dissolved particulate material) for the solvent preparation was negative, i.e., the carbonaceous residue of solvent extracted DPM was not mutagenic, while the surfactant preparation was positive, i.e., the particulate matter which is not dissolved by surfactant is, nevertheless, positive for mutagenic activity as a particulate dispersion in surfactant. Other tests of the surfactant preparation supernatant fraction using only centrifugation without subsequent filtration resulted in some activity in the surfactant preparation supernatant. This was interpreted as due to ultrafine particles of surfactant-dispersed DPM that were fully removed by filtration but not fully removed by centrifugation.

Chinese hamster pulmonary fibroblast-derived cell line (V79) was used to test for the induction of

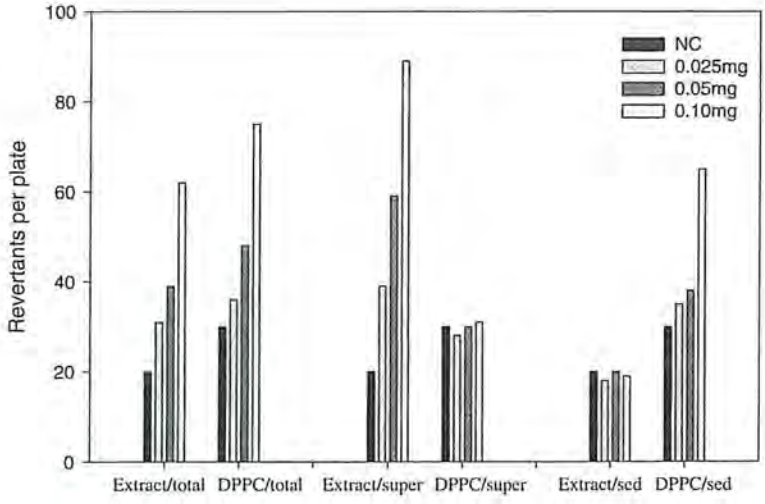

Figure 2. Mutagenic activity (TA98 *Salmonella typhimurium* TA98) versus DPM concentration: Mutagenic activity as number of revertant colonies is shown (y-axis) versus DPM concentration as solvent extract or DPPC surfactant dispersion. Activities are shown for the total solvent or surfactant preparation, for the filtered supernatant, and for the non-dissolved sediment. From: Wallace et al. (1987).

sister chromatid exchange (SCE) (Perry & Wolff, 1974), unscheduled DNA synthesis (UDS) (Mitchell et al., 1983) and chromosomal aberration (Preston et al., 1981). Results of the SCE assay as shown in Figure 3 (Keane et al., 1991) were similar to those of the Ames mutagenicity assay. Both organic extracted and surfactant-dispersed DPM materials induced SCE in V79 cells. After separation of the samples into supernatant and sediment fractions, the activity of both DPM preparations was found to reside in the supernatant fraction of the solvent-extracted samples, and in the sedimented fraction for surfactant dispersed samples.

Results of UDS assay are shown in Figure 4 (Gu et al., 1994). Both dispersions of DPM in surfactant and DMSO induced UDS in a concentration related manner. Again, induction of UDS was also found in the supernatant fraction of the DMSO-dispersed sample and in the sedimented fraction of the surfactant-dispersed sample. Chromosomal aberration (CA) studies found that surfactant-dispersed DPM was active for induction of CAs, generally increasing with DPM concentration (Gu et al., 2005).

Assay of induction of micronuclei (Lansne et al., 1984) using V79 cells and in Chinese hamster ovary-derived cells (CHO) was measured for DPM solvent extract and/or surfactant dispersion supernatant and particulate phase materials (Gu et al., 1992). The solvent supernatant (extract) of total samples after centrifugation and filtration, and the surfactant sediments (particulate material) from total sample centrifugation were active for micronucleus induction in CHO cells: the solvent extract was active in V79 cells, but the surfactant sediment was only marginally active in V79 cells (Figure 5).

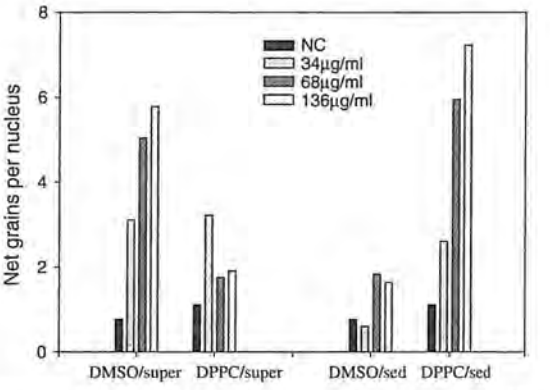

Figure 4. UDS (V79 mammalian cell) versus DPM concentration: UDS is represented as net autoradiographic grains/nucleus (y-axis) versus DPM concentration as solvent extract or DPPC surfactant dispersion; for total preparation, filtered supernatant, and sediment. From: Gu et al. (1994).

The results from these studies with different genetic endpoints in bacteria and in mammalian

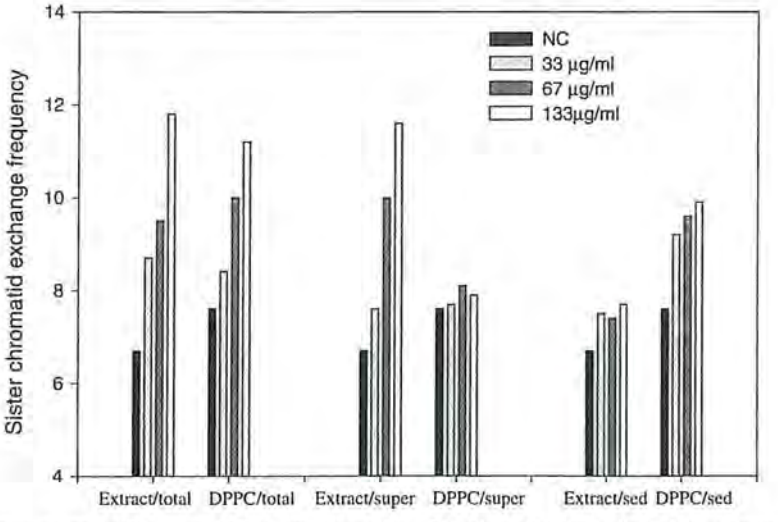

Figure 3. SCE (V79 mammalian cell) versus DPM concentration: Number of SCE/cell is shown (y-axis) versus DPM concentration as solvent extract or as DPPC surfactant dispersion, for the total preparation, filtered supernatant, and sediment. From: Keane et al. (1991).

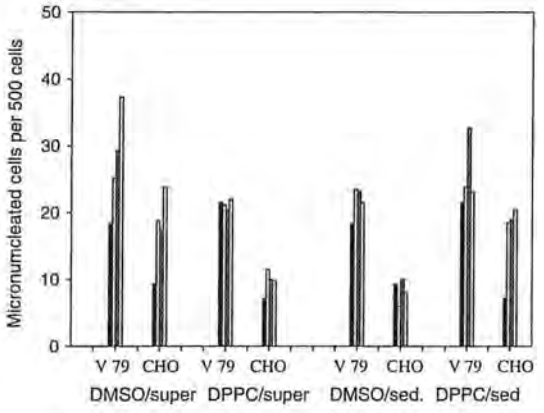

Figure 5. Micronucleus formation (V79 mammalian cells) versus DPM concentration: Micronucleus frequency per 500 cells (y-axis) is shown versus DPM concentration as solvent or surfactant filtered supernatant or sediment. From: Gu et al. (1992).

cells are consistent and show that DPM genotoxic activities are expressed when the DPM is dispersed into DPPC surfactant; and those activities are associated with the non-dissolved particulate phase material. DPPC dispersion does not extract genotoxicants from the DPM particles; rather, the phospholipid coats and "solubilizes" (not "dissolves") the DPM, providing a hydrophilic coating and permitting the dispersion of the surfactant-coated DPM in aqueous media. These findings indicate that genotoxic activity and potential carcinogenicity associated with DPM inhaled into the lung may be made bioavailable by virtue of the solubilization/dispersion properties of pulmonary surfactant components.

Fine respirable mineral particle surface nanostructure and disease risk

Mineral particle composition can strongly affect toxic activity and disease risk associated with respirable dust exposures. One of the most studied mineral dusts is respirable crystalline silica, e.g., quartz, a known potent agent for pulmonary fibrosis (Green & Vallyathan, 1995), and evaluated by the International Agency for Research on Cancer (IARC) to be a human carcinogen under some exposure conditions. Quartz dust also expresses some much higher in vivo toxicities in animal models than a number of other mineral dusts and other respirable materials when the dose metric is surface area. However, even beyond this mineral specificity, quartz dust-induced human disease risk is affected significantly by sub-micrometer scale surface coatings by heteroatomic materials. Seemingly anomalous differences in lung fibrosis disease risk from silica dust exposures in mixed dust composition atmospheres have been observed, e.g., in coal workers' pneumoconiosis (Attfield & Morring, 1992), and these anomalies have been associated with the existence of sub-micrometer thick mineral coatings or "occlusion" of silica particles by aluminosilicate. In animal model experiments, some workplace silica dusts were found to be much diminished in fibrogenic activity compared to the dusts after acid-etching, suggesting a prophylactic surface coating on the silica particles, e.g., of aluminosilicate clay (Le-Bouffant et al., 1982). Spectroscopic analyses have shown such sub-micrometer thick aluminosilicate coating or "occlusion" of respirable silica particles, detected by contrasting scanning electron microscopy - energy dispersive X-ray analysis of individual silica particle composition at high and at low electron beam energies to probe particle composition with depth (Wallace et al., 1990b, 1992, 1994; Wallace & Keane, 1993; Hnizdo & Wallace, 2002). Such sub-micrometer coatings or occlusion of quartz particle surfaces by clay have been associated with the epidemiological "coal rank anomaly" in the prevalence of coal workers' pneumoconiosis (Walton et al., 1971; Robock & Klosterkotter, 1973; Kreigseis & Scharmann, 1982; Attfield & Morring, 1992). Research indicated that the fraction of silica particles surface occluded by aluminosilicate decreased with increasing coal rank, with a consequent greater fraction of silica particles with "biologically available" surface for dusts from mines of higher rank coals (Harrison et al., 1997). The equivalent was seen in a study of anomalous differences in silicosis risk between Chinese metal mine workers and pottery workers quantified in a silicosis medical registry of some 20,000 workers (Chen et al., 2005). Normalizing the workers' cumulative respirable silica dust exposures to cumulative respirable "surface-available" silica dust, i.e., the fraction of respirable silica dust particles not surface occluded by clay aluminosilicate, resolved much of the difference in risk (Harrison et al., 2005).

Nano-scale surface composition and structure were found to be important for dust toxicity and

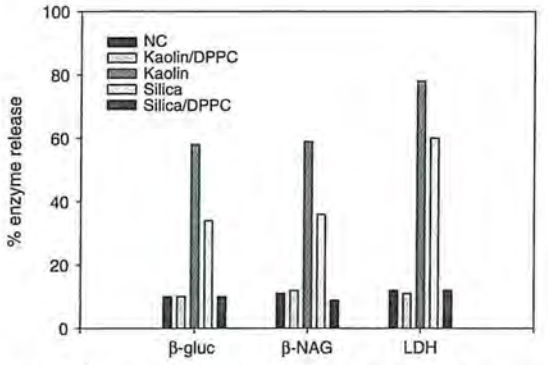

Figure 6. Quartz and kaolin expression of in vitro cytotoxicities: Cytotoxicities are shown (*y*-axis) as % release of cellular cytosolic enzyme LDH , and cellular lysosomal enzymes beta-glucuronidase, and beta-*N*-acetyl glucosaminidase. Native quartz and kaolin dusts are comparably cytotoxic on a surface area basis; and both have their toxicities fully suppressed immediately after incubation with DPPC surfactant. From: Wallace et al. (1988).

hazard in a "hard metal" manufacturing workplace, e.g., producing tungsten carbide (WC) grit cemented together by cobalt metal to form hard sharp edges for cutting tools. Surface analyses by scanning Auger spectroscopy in combination with scanning electron microscopy – X-ray spectroscopy (Stephens et al., 1998) found nano-meter thin cobalt coating on respirable WC particles generated in a new hard metal production facility where sentinel adverse health effects had been observed. The structure of those coatings, i.e., thin Co in contact with the underlying WC particle surface, resulted in heightened levels of catalytically generated reactive oxygen species in aqueous media; this led to associated exacerbation of *in vitro* toxicities in comparison to dusts from a conventional fabrication process (Keane et al., 2002a, b).

Surfactant effects on mineral particle toxicity

Exposure to respirable aluminosilicate kaolin clay dust does not present the high risk of lung fibrosis presented by quartz dust; and aluminosilicate surface coatings on quartz particles are associated with diminished silicosis risk. Nevertheless, kaolin dust expresses *in vitro* cytotoxic activities comparable to that of quartz dust on a surface area basis, as measured by mammalian cell release of LDH and lysosomal enzymes or by erythrocyte membranolysis (Figure 6) (Vallyathan et al., 1988; Wallace et al., 1989, 1992). Thus, aluminosilicate dust false positives prevent the effective use of short term *in vitro* assays to predict disease hazard. This suggests that, similarly, *in vitro* cytotoxicity assays of NP may not be directly predictive of disease risk. Again, respirable particle condition-

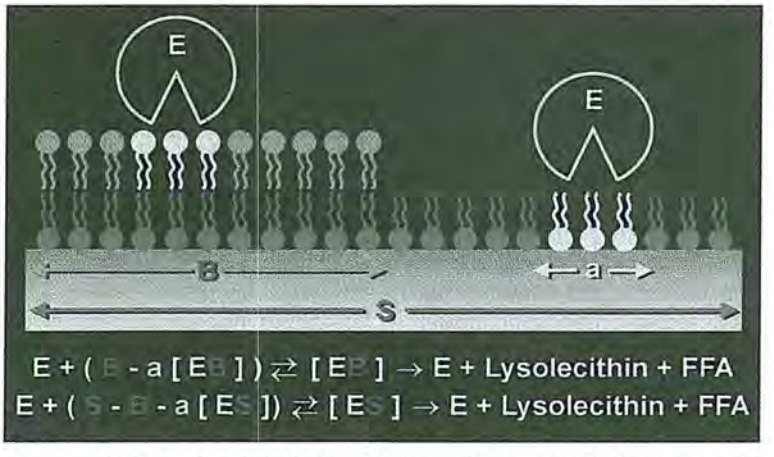

Figure 7. Bi-layer model of phospholipase digestion of phospholipid surfactant adsorbed on a mineral particle surface: An inner layer of DPPC molecules (S) adsorb to hydrophilic mineral dust surface; this is backed by a DPPC layer oriented in the reverse direction (B). Phospholipase A_2 enzyme (E) digests the outer layer with a rapid and non-mineral-specific rate, producing lyso (monoacyl) phosphatidylcholine and free palmitic fatty acid. Enzyme access to the inner layer is partially hindered by the outer layer of DPPC (B), by the presence of adjacent enzymes (a), and by mineral-specific surface functional groups interacting with adsorbed DPPC, e.g., DPPC phosphate binding to clay surface aluminol, to result in different conformations of the adsorbed DPPC with consequent differences in rates of removal and restoration of particle toxicity. From: Wallace et al. (1994b).

ing by pulmonary surfactants will occur upon deposition of mineral particles in the deep lung (Emerson & Davis, 1983). DPPC is adsorbed from dispersion in physiological saline by quartz (Jaurand et al., 1979) or by kaolin (Wallace et al., 1975, 1985) and suppresses the dusts' otherwise prompt *in vitro* cytotoxicity.

However, *in vitro* assays of mineral dusts including modeling of surfactant conditioning do not provide unambiguous prediction of disease risk; e.g., surfactant treatment suppresses the toxicity of quartz for a period of time, presenting an apparent false negative assay result. Thus the next in vivo event must be considered: the cellular uptake and enzymatic digestion of the particles with possible hydrolysis and removal of prophylactic surfactant from the particles and consequent restoration of toxicity over time. In acellular and *in vitro* cellular studies, phospholipase A2 (PLA2) enzyme digests DPPC from the dusts, with restoration of cytotoxic activity. Kinetics of the process is well-modeled as a two-exponential function process with an outer layer of DPPC molecules rapidly hydrolyzed at the glycerol ester linkages by the lipase, while the inner DPPC layer in direct contact with the mineral surface is digested more slowly and with mineral specificity (Figure 7). For extracellular PLA2 acting at neutral pH, the inner layer DPPC is digestively removed much more slowly from kaolin than from quartz (Wallace et al., 1988, 1992; Hill et al., 1995; Liu et al., 1996, 1998; Das et al., 2000; Keane et al., 1990, 2005). This is associated with mineral-specific conformations of adsorbed DPPC (Murray et al., 2005) in which interaction of the DPPC phosphate with kaolin surface aluminol groups confers an added steric hindrance to the PLA2 hydrolytic activity on the carbonyl ester adjacent to the phosphate. This difference in rates for surfactant removal is seen for quartz versus kaolin particles phagocytosed by pulmonary macrophage- derived cells e.g., under conditions in the acidic pH phagolysosome (Figure 8); but kaolin also is stripped of surfactant and restored to activity, albeit after a longer assay incubation of several days (Keane & Wallace, 2005). This suggests that a predictive assay would require the use of cell systems modeling the neutral pH phagolysosomal systems of interstitial cells (Adamson et al., 1989; Johnson & Maples, 1994).

DPPC is a limited model of lung surfactant, and of other biological molecules that may be found in the lung alveolar hypophase. Mineral specificity of prophylaxis and rate of restoration of expression of toxicity may be affected additionally by other lipids or lipoproteins. Two sizes of industrial ultrafine carbon blacks were found to adsorb significant DPPC and Surfactant Protein-D from aqueous dispersion, affecting particle agglomeration and precipitation; while little effect was noted for their incubation with fibrinogen or albumin (Kendall et al., 2004). Lipoprotein fractions of cell test system media serum can reduce the expression

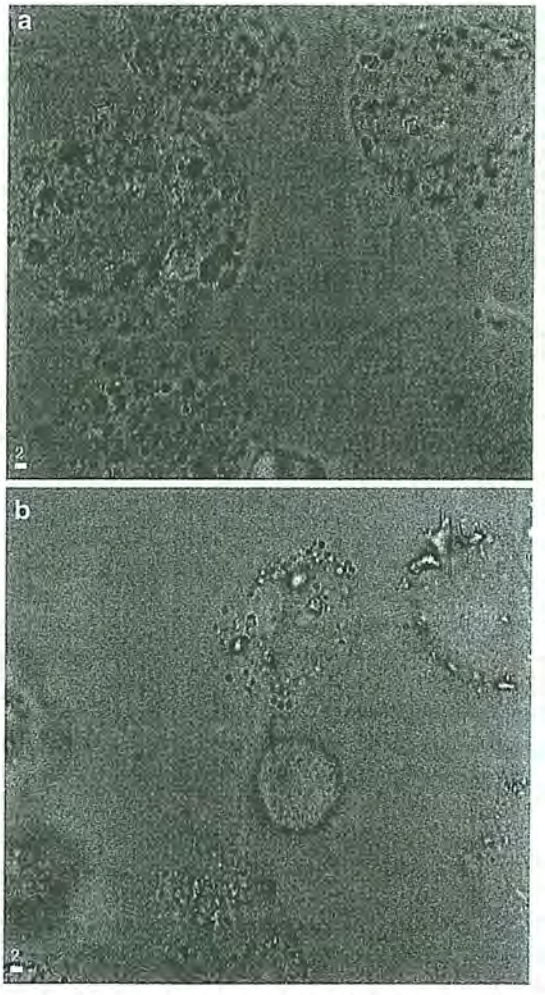

Figure 8. Removal of fluorescent-labeled phospholipids on kaolin (a) or quartz (b): Fluorescence microscopy images phospholipid remaining on phagocytosed quartz or kaolin particles at 8 Days after challenge. A green fluorescent boron-labeled analogue of diacyl phosphatidylcholine is shown retained on kaolin particles in cells (a), but lost from silica particles (b) with associated expression of cell toxicity.

of crystalline silica cytotoxicity (Kozin & McCarty, 1977; Barrett et al., 1999a, b), with reactivation following trypsin digestion (Fenoglio et al., 2005). Quartz and kaolin dust prompt *in vitro* induction of LDH release from macrophage is suppressed in 10% fetal bovine serum (FBS) medium; however, quartz but not kaolin activity was restored at 6 h (Gao et al., 2000, 2001, 2002). This indicates that short-term *in vitro* assay results can be affected by assay system nutrients that are not necessarily representative of in vivo pulmonary hypophase exposures. However, in vivo acute inflammatory reactions are accompanied by increased permeability of the microvasculature with transudation of plasma protein, including albumin, (Slauson & Cooper, 1990; Driscoll, 1994, 1996) into the lung alveoli. Thus, following non-mineral specific response of alveolar macrophages to quartz or kaolin, the subsequent inflammatory response may provide a second-tier and mineral specific prophylaxis to deposited particles, conditioning their subsequent interstitial interactions.

Lessons for NP studies

Diesel exhaust nanoparticulate material and respirable micrometer-sized mineral dust expression of *in vitro* cytotoxicity or genotoxicity can be strongly affected by particle surface conditioning by a phospholipid component of lung surfactant, modeling an initial in vivo phenomenon not usually considered for assays of respirable particle toxicities. Detailed surface structural features not considered in conventional industrial hygiene characterizations can significantly affect disease risk associated with respirable mineral dust exposures. NP have high specific surface areas. Therefore, particle surface composition and its pulmonary surfactant conditioning should be considered in the design and interpretation of *in vitro* cytotoxicity or genotoxicity assays of NP, and for in vivo assays which might involve disruption of the pulmonary hypophase in the lung of the animal model, e.g., in some localized regions during instillation challenge.

Experience with diesel NP suggests that surfactant conditioning may provide a physiologically plausible method for assay of other carbonaceous or organic NP material potential for genotoxic activity under conditions modeling particle deposition in the deep lung. Research has shown that DPPC, a major phospholipid component of lung surfactant, does not extract genotoxicant compounds from diesel exhaust NP; rather, the surfactant coats the particles and those non-dissolved but surface-conditioned particles are able to express genotoxic activity. This has been demonstrated for bacterial mutagenicity and for mammalian cell DNA and clastogenic damage in a number of specific assay endpoints, e.g., SCE, UDS, chromosomal aberrations, micronucleus induction, and single cell gel electrophoresis-visualized DNA strand breaks. The amount of surfactant used in pre-conditioning the NP should be in excess of that required for bilayer surface coverage of the NP material. Measures of *in vitro* membranolytic activity suppression versus amount of surfactant adsorption on respirable quartz and kaolin dusts indicate this is approximately 5 mg DPPC per square meter of dust surface, the dust surface independently measured by nitrogen gas adsorption isotherms (Wallace et al., 1992). However, results indicate that additional DPPC will adsorb to several additional molecular layers on the dusts. In the case of diesel exhaust NP there is evidence that surfactant amounts on the order of equal mass of DPPC per mass of DPM are required for maximum expression of toxicity (Wallace et al., 1987). This may reflect extremely high specific surface areas of some DPM for surfactant adsorption, or a preparative effect of surfactant dispersion concentration on the agglomerative state of sampled material.

Such surfactant surface conditioning of inorganic respirable particles does not immediately provide a similarly direct and effective method for assay of non-genotoxic disease hazard; and these caveats may apply also to NP. In the absence of surfactant surface conditioning, a false positive assessment can result from the innate surface toxicity of some minerals; that is, toxic surface interactions observed *in vitro* are not manifest as in vivo pathology due to physiologic prophylaxis systems in the lung. For instance, assay of alumina or aluminosilicate NP in the absence of physiological surfactant or serum pre-conditioning risks the false positive results (for prediction of fibrosis) predicted by *in vitro* membranolysis and cytokine release assays of micrometer-sized respirable kaolin dust. The converse problem also is possible: short term assays can give a false

negative result because of physiological but transient prophylaxis by surfactant conditioning of toxic dusts, whose true hazard might be revealed by longer assay challenge times, e.g., even quartz or other hazardous dust expression of toxic activity may be delayed several days in vivo and *in vitro* by surfactant surface conditioning. And the use of supplemented serum to nourish cells in longer-term *in vitro* assays can risk a false negative assay result from prophylaxis effects of nutrient components not representative of the in vivo milieu. There is a general caveat to the use of DPPC or any simplified model of lung surfactant for such surface conditioning for bioassay: lung surfactant is a complex lipoprotein mixture and the composition of potentially prophylactic biomolecules in the alveolar hypophase can change with exposure, e.g., with inflammation-associated transudation of albumin or other serum constituents into the lung. Thus the adsorption and possible resultant prophylactic effects of these different biomolecular components of the lung hypophase may differ, perhaps with different particle surface specificities.

Research on surfactant and serum interactions with respirable particle surfaces has indicated profound effects on the expression of toxicity suggesting that interactions of respired NP with biological molecular constituents of the hypophase liquid lining of the lung should be considered in the preparation and interpretation of bioassays of potential NP respiratory hazard.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Andrew D. Maynard
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