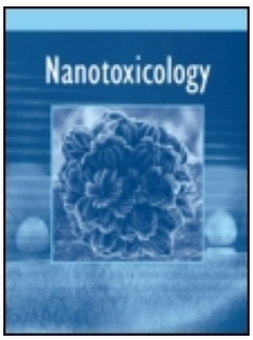

Nanotoxicology

ISSN: 1743-5390 (Print) 1743-5404 (Online) Journal homepage: http://www.tandfonline.com/loi/inan20

Improved method to disperse nanoparticles for *in vitro* and *in vivo* investigation of toxicity

Tina M. Sager, Dale W. Porter, Victor A. Robinson, William G. Lindsley, Diane E. Schwegler-Berry & Vincent Castranova

To cite this article: Tina M. Sager, Dale W. Porter, Victor A. Robinson, William G. Lindsley, Diane E. Schwegler-Berry & Vincent Castranova (2007) Improved method to disperse nanoparticles for *in vitro* and *in vivo* investigation of toxicity, Nanotoxicology, 1:2, 118-129, DOI: 10.1080/17435390701381596

To link to this article: https://doi.org/10.1080/17435390701381596

Nanotoxicology, June 2007; 1(2): 118–129

informa
healthcare

Improved method to disperse nanoparticles for *in vitro* and *in vivo* investigation of toxicity

TINA M. SAGER[1,2], DALE W. PORTER[1,2], VICTOR A. ROBINSON[1], WILLIAM G. LINDSLEY[1], DIANE E. SCHWEGLER-BERRY[1], & VINCENT CASTRANOVA[1,2]

[1]*Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia &* [2]*Department of Physiology and Pharmacology, West Virginia University School of Medicine, Morgantown, West Virginia, USA*

Abstract
Nanoparticles agglomerate and clump in solution, making it difficult to accurately deliver them for *in vivo* or *in vitro* experiments. Thus, experiments were conducted to determine the best method to suspend nanosized particles. Ultrafine and fine carbon black and titanium dioxide were suspended in phosphate buffered saline (PBS), rat and mouse bronchoalveolar lavage fluid (BALF), and PBS containing dipalmitoyl phosphatidylcholine (DPPC) and/or mouse serum albumin. To assess and compare how these various suspension media dispersed the nanoparticles, images were taken using light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The results of this study show that PBS is not a satisfactory medium to prepare nanoparticle suspensions. However, BALF was an excellent media in which to suspend nanoparticles. The use of PBS containing protein or DPPC alone, in concentrations found in BALF, did not result in satisfactory particle dispersion. However, PBS-containing protein plus DPPC was satisfactory, although less effective than BALF.

Keywords: *Nanoparticles, agglomeration, dispersion, titanium dioxide, carbon black, bronchoalveolar lavage fluid*

Introduction

Human exposure to airborne ultrafine or nanosized particles (particles less than 100 nm in aerodynamic diameter (Donaldson et al. 2002)) has increased dramatically over the last century due to anthropogenic sources. The rapidly developing field of nanotechnology is deemed likely to become another source of human exposure (Oberdörster et al. 2005b). Nanotechnology is on track to far exceed the impact of the Industrial Revolution and is projected to become a $1 trillion market by 2015. Engineered nanoparticles are currently being used in the manufacturing of a variety of commercial products, such as sporting goods, tires, stain-resistant clothing, sunscreens, cosmetics, and electronics (Nel et al. 2006). However, there is sufficient 'ultrafine particle' toxicology literature to indicate that the large and diverse group of manufactured nanoparticles arising from nanotechnology could comprise an inhalation hazard of unknown potential (Donaldson et al. 2006). Therefore, determining a protocol to assess the toxicity of nanosized particles is of great importance in defining and understanding the health effects associated with nanoparticle exposure.

Investigation of nanoparticles has shown that they possess two characteristics which promote their toxicity. The first important characteristic involves physico-chemical properties of the nanoparticle, which includes the surface area of the nanoparticle, the smaller the particle the greater the surface area; the second characteristic is the ability of the nanoparticle to traverse cell membranes (Seaton & Donaldson 2005). Therefore, particle size and surface area are important characteristics from a toxicological perspective. As the size of the particle decreases, its surface area increases. This increase in surface area allows a greater proportion of its atoms or molecules to be displayed on the surface rather than the interior of the nano-material (Nel et al. 2006). For this reason, many recent studies have examined whether the mass of particles or surface area of particles should be used as the dose metric to assess pulmonary toxicity of a specific nanoparticle.

Previous unpublished studies conducted in our laboratory have shown that when cells are

Correspondence: Vincent Castranova, M/S 2015, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505, USA. Tel: +1 304 285 6032. Fax: +1 304 285 5938. E-mail: vic1@cdc.gov

ISSN 1743-5390 print/ISSN 1743-5404 online
DOI: 10.1080/17435390701381596

administered a dose of coarse or fine silica based on mass, the fine particles have greater toxicity. However, when the doses are equalized based on surface area (instead of mass) the toxicity of the two particle sizes of silica are nearly identical, indicating that perhaps the surface area rather than mass is the more appropriate metric of dose for pulmonary toxicity studies. In addition, an *in vivo* study using ultrafine carbon black (UFCB) and fine carbon black (FCB) was conducted to assess whether particle mass or surface area should be used as a particle toxicity indicator. Intratracheal instilled UFCB particles were found to be more toxic on an equivalent mass basis than the FCB. However, when dose was normalized to equivalent particle surface area, the FCB particles were more toxic than the UFCB. These unexpected outcomes lead to an examination of the nanoparticle suspensions used in these experiments to address dispersion and agglomeration issues. Both light and electron microscopy indicated that dispersion of UFCB in phosphate-buffered saline (PBS) was poor and resulted in agglomerates in the micrometer size range. Therefore, surface area measured by gas absorption may have far exceeded the biologically available surface of an agglomerate.

Particle agglomeration and inadequate dispersion are both important obstacles encountered in toxicology studies dealing with nanomaterials. Nanoparticles agglomerate and clump in solution, making it difficult to accurately deliver nano-sized particles in an *in vivo* or *in vitro* experimental procedure. When nanoparticles agglomerate, their deposition characteristics change because the agglomerate has a greater diameter than the singlet particles (Donaldson et al. 2001). Delivering agglomerated nanoparticles in an *in vitro* or *in vivo* experimental situation could actually lead to an inaccurate assessment of that specific nanoparticle's toxicity. This outcome could lead to the conclusion that the nanoparticle was less toxic than it actually is. For example, intratracheal instillation of poorly dispersed single-walled carbon nanotubes to rats caused asphyxiation and the absence of a dose-dependent response (Warheit et al. 2004). The authors concluded that these pulmonary responses were artifacts of instillation of large agglomerates and were not representative of workplace exposures. In contrast, Shvedova et al. (2005) demonstrated that better dispersion of single-walled carbon nanotubes by aspiration resulted in a dose-dependent and progressively diffuse interstitial fibrosis associated with deposition of more dispersed carbon nanotube structures.

Due to the particle agglomeration and dispersion problems limiting nanoparticle toxicology research, experimentation is required to develop more accurate ways to prepare nanoparticle suspensions. To address the nanoparticle agglomeration and dispersion problems, experiments were conducted to determine the best method to suspend nanosized particles. It was hypothesized that upon inhalation, nanosized particles would contact the alveolar fluid lining of the lung, which is composed of a mixture of alveolar surfactant and protein. The interaction of the particles with the alveolar surfactant and proteins may play a role in dispersing the particles, keeping them from agglomerating once they are deposited in the lung. Therefore, the goal of the present investigation was to examine the dispersion capabilities of bronchoalveolar lavage fluid (BALF) or its components and to determine if such treatment altered the surface activity of particles.

A series of *in vitro* and *in vivo* dispersion experiments have been conducted to aid in establishing a more reproducible protocol for making nanosized particle suspensions. Specifically, ultrafine and fine carbon black and titanium dioxide (TiO_2) were suspended in a variety of suspension media including phosphate buffered saline (PBS), rat and mouse bronchoalveolar lavage fluid (BALF), dipalmitoyl phosphatidylcholine (DPPC), albumin, or the combinations of DPPC + albumin. To assess and compare how well the various suspension media dispersed the particles, images were taken using light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Also, a series of *in vivo* and *in vitro* studies assessing inflammatory potential and cytotoxicity were done using silica, a particle of known toxicity, to examine whether coating the nanoparticles with the BALF would attenuate toxicity of the nanoparticles.

Methods

Animals

The rats used for BALF collection for these experiments were male Sprague-Dawley (Hla: (SD) CVF) rats weighing 200–300 g ($\sim$ 10 weeks old at arrival) obtained from Hilltop Lab Animals (Scottsdale, PA, USA). The mice used for BALF collection were C57BL/6J mice ($\sim$ 8 weeks old at arrival) obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). The animals were housed in an AAALAC-accredited, specific pathogen-free, environmentally controlled facility. The animals were monitored to be free of endogenous viral pathogens, parasites, mycoplasms, Helicobacter and CAR Bacillus. Animals were housed in ventilated cages which were provided HEPA-filtered air, with Alpha-Dri virgin cellulose chips and hardwood Beta-chips used as bedding. The rats were maintained on a ProLaB 3500 diet and tap water, both of which were provided

ad libitum. The mice were maintained on a Harlan Teklad 7913, 6% fat, irradiated diet and tap water, both of which were provided *ad libitum.*

Bronchoalveolar fluid collections

Rats were euthanized with an i.p. injection of sodium pentobarbital (>100 mg/kg body weight) and exsangainated by cutting the descending aorta. A tracheal cannula was inserted and bronchoalveolar lavage was conducted (Porter et al. 2002). A 6 ml aliquot of cold Ca^{+2} and Mg^{+2} free phosphate-buffered saline (PBS) was used for the lavage wash. The cold PBS was flushed into and out of the lungs two times before the lavage fluid was collected. The BALF from five rats was combined and then placed in a centrifuge at 600 ***g*** for 10 min using a Sorvall RC 3B Plus centrifuge (Sorvall Thermo Electron Corporation, Asheville NC, USA). The supernatant was decanted into a new tube while the pellet was discarded. This lavage fluid was then used as the vehicle for particle suspensions. The BALF was collected fresh the same day that the particulate suspensions were made.

Bronchoalveolar lavage of mice was conducted as previously described by our laboratory (Zeidler et al. 2004). Mice were weighed (mean body weight 24.0 g) and euthanized with an intraperitoneal injection of sodium pentobarbital (>100 mg/kg). The trachea was cannulated with a blunted 22-gauge needle, and bronchoalveolar lavage (BAL) was performed using cold sterile Ca^{+2} and Mg^{+2} free PBS at a volume of 0.6 ml. BALF was centrifuged at 600 ***g*** for 10 min, and the supernatant collected.

Particle sieving

Ultrafine carbon black (Printex 90, 14 nm), fine carbon black (Arosperse 15V, 260 nm) and ultrafine titanium dioxide (Aeroxide TiO_2 P-25, 21 nm) were all obtained as a gift from the Degussa Corporation (Parisppany, NJ, USA). Fine TiO_2 (titanium (IV) oxide, 1 μm) was purchased from Sigma-Aldrich (Atlanta, GA, USA).

Each particulate sample was individually sieved in a Retsch AS 200 Sieve (Retsch GmbH, Haan, Germany) at a vibration amplitude of 50 for 15 min to break apart large clumps. Three different sieve sizes were used successively in the sieving process. Each particle was first loaded onto a standard testing sieve with 1.18 mm openings. The second sieve had 250 μm openings. The third sieve had 45 μm openings. The particles that were able to pass all the way through the third sieve (45 μm openings) during the 15-min sieving process were collected and used for the particle dispersion experiments.

Suspension of UFCB, FCB, $UFTiO_2$ and $FTiO_2$ in PBS, rat BALF, or DPPC

Ultrafine carbon black (UFCB, 5.0 mg/ml), fine carbon black (FCB, 40.83 mg/ml), ultrafine titanium dioxide ($UFTiO_2$, 3.47 mg/ml), and fine titanium dioxide ($FTiO_2$, 8.93 mg/ml) were suspended in PBS, rat BALF, or dipalmitoyl phosphatidylcholine (DPPC, 160 μg/ml). The DPPC concentration used reflected the average phospholipid content present in BALF of control air exposed rats (Porter et al. 2001). The DPPC used for the particle dispersion experiments was suspended in PBS and sonicated at 10% duty cycle for 15 min.

Each previously sieved particle sample was weighed out to obtain the desired concentration. Each respective particle was then suspended in one of the three suspension media at a ratio to obtain the desired concentration. Once the particles were added to the suspension media, the suspensions were pulse sonicated with 5 individual pulses at a duty cycle setting of 10% and an output setting of 1 with a Branson 450 Sonifier probe sonicator (Branson Ultrasonics Corporation, Danbury CT, USA).

Suspension of UFCB in PBS, mouse BALF, or dispersion media

UFCB (0.8 mg/ml) was suspended in PBS, mouse BALF, or one of the following dispersion media: (i) PBS containing 10 μg/ml DPPC, (ii) PBS containing 0.6 mg/ml BSA, or (iii) PBS containing a DPPC/BSA combination. DPPC was prepared as a 10 mg/ml stock solution in absolute ethanol. Thus, the final concentration of ethanol in the two dispersion media was 0.1% (v/v).

Suspensions were sonicated using Branson 450 Sonifier probe sonicator with a duty cycle setting of 10% and an output setting of 1. Samples were sonicated either with 5 individual pulses or continuously for 10 min.

Light microscopy

After the particle suspensions were sonicated, a 25 μl sample was placed onto a precleaned Superfrost/Plus microslide (Daigger, Wheeling, IL, USA). The samples were covered. The samples were then viewed at both 10× and 40× using an Olympus AX70 photomicroscope (B&B Microscopes, Pittsburgh,

PA, USA) and a Sony 3CCD color video camera, DXC9000, capturing the images with SimplePCI software (Compix Inc., Imaging Systems, Cranberry Township, PA, USA).

Particle suspension preparation for SEM and TEM analysis

For the SEM and TEM analysis, each particle type was suspended as previously described. A 1 ml sample of the suspension was loaded into a syringe. Samples were then aerosolized with an atomizer (Model 3076, TSI, Shoreview, MN, USA) using dry filtered air at 24 kPa (35 psi). The sample suspension was injected into the atomizer using a syringe pump (Universal Model 575, Valley Scientific, Walpole, MA, USA) at a flow rate of 1 ml/min. Excess sample was not recirculated after atomization. Samples were collected immediately after atomization on 25 mm polycarbonate membrane filters (Nucleopore, Whatman, Florham Park, NJ, USA) using a vacuum source at a flow rate of 3 l/min. The aerosol was not dried prior to collection on the filter.

For the scanning electron microscopy analysis, the filters were trimmed, and one quarter was placed on an aluminum stub with double-stick carbon tape. The sample was then sputter coated with gold/palladium and viewed with a JEOL 6400 scanning electron microscope. For transmission electron microscopy analysis, the particles were diluted in double distilled filtered water, and a drop was placed on a formvar-coated copper grid to dry. The sample was viewed with a JEOL 1220 transmission electron microscope.

Comparison of toxicity of Min-U-Sil 2 quartz suspensions in PBS, DPPC, and BALF

In vivo *exposures*. BALF from rats was obtained as described above. Next, crystalline silica (Min-U-Sil 2 silica; Berkley Springs, WV, USA) was suspended in either PBS or BALF. Rats were exposed to the different silica suspensions via intratracheal instillation (IT) at a dose of 20 mg/rat as described previously by our laboratory (Porter et al. 2002). At 24 h post-IT, the animals were euthanized with an i.p. injection of sodium pentobarbital (>100 mg/kg body weight), and brochoalveolar lavage was conducted. Using these lavage samples, cell counts (polymorphonuclear leukocytes and alveolar macrophages) were conducted to assess inflammation, and lactate dehydrogenase (LDH) activity was measured to assess cellular injury. Cell counts were conducted using an electronic cell counter (Beckman Coulter Multisizer 3 Counter, Haleah, FL, USA) and LDH activity was measured using Roche COBAS MIRA Plus chemical analyzer (Roche Diagnostic Systems Inc., Branchburg, NJ, USA) as described previously by our laboratory (Porter et al. 2002).

In vitro *exposures of A549 cells*. A549 cells were purchased from the American Type Culture Collection (ATCC # CCL-185) and cultured in Hams F-12K media with 2 mM L-glutamine. Fetal bovine serum (10%) was added to the media along with penicillin/streptomycin (10 units/10 ug/ml) and amphotericin B (0.25 ug/ml) to produce a complete medium for propagation of the cells. Cells were maintained in 75 or 150 cm^2 culture flasks and passaged until the cell monolayer covered approximately 80% of the surface area of the flask. Cells from passages number 2 to 10 only were used in dust exposure experiments.

Prior to particulate exposure, cells were plated at 1.5×10^5 cells/ml in 12-well plates, with 1.0 ml per well using the above mentioned complete medium minus phenol red. One day later, the medium was removed and replaced with serum-free media containing Min-U-Sil 2 silica particles suspended in PBS, BALF or DPPC at logarithmically spaced concentrations ranging from 0.01–1.0 mg/ml. At 18 h post-exposure, the media were removed from the wells and centrifuged at 500 ***g*** for 10 min to pellet any cells or dust particles. Following centrifugation, 200 µl of each supernatant was removed for measurement of LDH activity.

Statistics

Statistical differences for the *in vivo* experiments examining the toxicity of silica suspended in PBS, BALF, or DPPC were determined using a one-way analysis of variance (ANOVA) with significance set at $p \leq 0.05$. Individual means were compared using the Tukey pairwise multiple comparison procedure with an overall significance level of 0.05 (Figure 9 and 10). Statistical differences for the *in vitro* experiments examining the toxicity of silica suspended in PBS, BALF, or DPPC were determined using an unpaired t-test with significance set at $p \leq 0.05$ (Figure 11).

Results

Comparison of UFCB and FCB suspensions in PBS, DPPC, and rat BALF

The UFCB particles suspended in the different media were viewed using light microscopy.

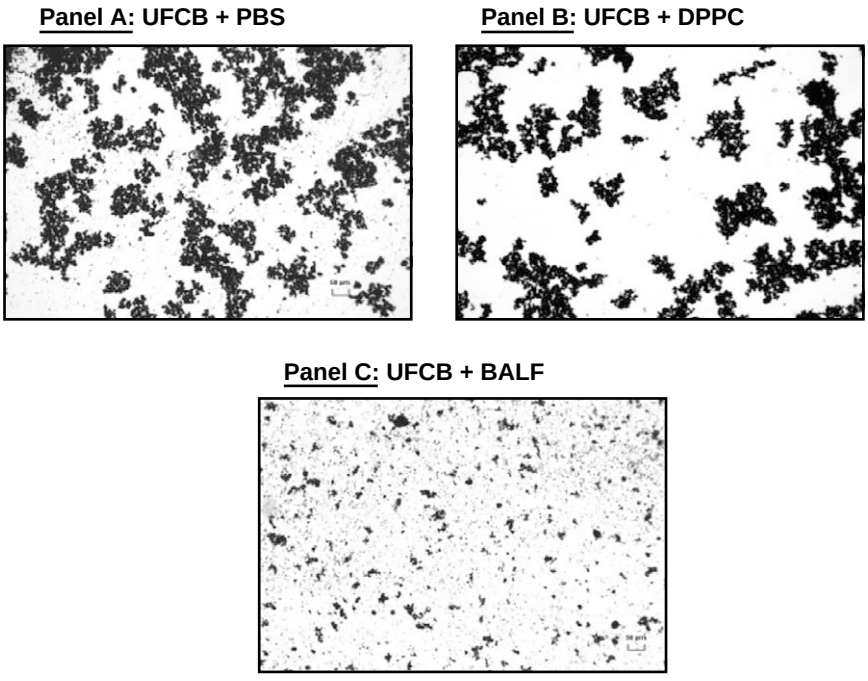

Figure 1. Light microscopy evidence of improved dispersion of ultrafine carbon black (UFCB) by bronchoalveolar lavage fluid (BALF). *Panel A*: UFCB (5.0 mg/ml) suspended in PBS. *Panel B*: UFCB (5.0 mg/ml) suspended in DPPC (160 μg/ml). *Panel C*: UFCB (5.0 mg/ml) suspended in rat BALF. Each particle suspension was sonicated with 5 individual pulses, and then a 25 μl sample was viewed using light microscopy at a magnification of 10 ×.

Upon examination of the 5 mg/ml of UFCB, the UFCB suspended in PBS had large agglomerates (Figure 1, panel A). The UFCB suspended in the DPPC/PBS mixture (Figure 1, panel B) had fewer agglomerates than the UFCB+PBS; however, the agglomerates present were comparable in size to the UFCB+PBS agglomerates. In contrast, the UFCB suspended in rat BALF (Figure 1, panel C) had

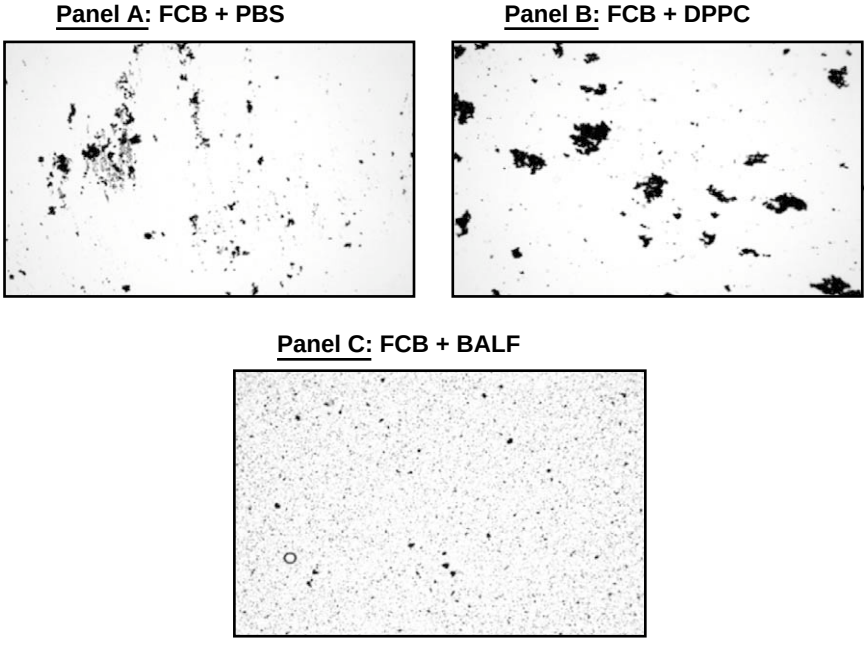

Figure 2. Light microscopy evidence of improved dispersion of fine carbon black (FCB) by bronchoalveolar lavage fluid (BALF). *Panel A*: FCB (40.83 mg/ml) suspended in PBS. *Panel B*: FCB (40.83 mg/ml) suspended in DPPC (160 μg/ml). *Panel C*: FCB (40.83 mg/ml) suspended in BALF. Each particle suspension was sonicated with 5 individual pulses, and then a 25 μl sample was viewed using light microscopy at a magnification of 10 ×.

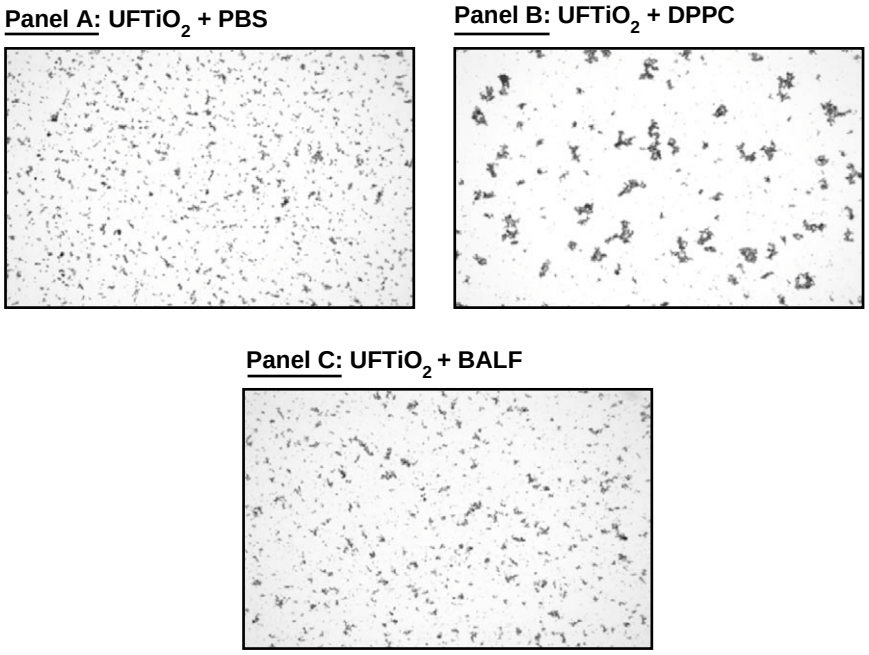

Figure 3. Light microscopy evidence of improved dispersion of ultrafine titanium dioxide ($UFTiO_2$) by bronchoalveolar lavage fluid (BALF). *Panel A*: 3.47 mg/ml of $UFTiO_2$ suspended in PBS. *Panel B*: 3.47 mg/ml of $UFTiO_2$ suspended in DPPC (160 μg/ml). *Panel C*: 3.47 mg/ml of $UFTiO_2$ suspended in BALF. Each particle suspension was sonicated with 5 individual pulses, and then a 25 μl sample was viewed using light microscopy at a magnification of 10 ×.

significantly smaller and fewer agglomerates and exhibited a more uniform dispersion pattern.

Upon examination of the 40 mg/ml FCB suspensions, the FCB suspended in PBS (Figure 2, panel A) showed moderate particle agglomeration and a non-uniform dispersion pattern.

The suspension of FCB in DPPC (Figure 2, panel B) had noticeably larger agglomerates in comparison to the FCB+PBS suspension. The FCB suspended in BALF (Figure 2, panel C) had noticeably fewer agglomerates than either the FCB+PBS or the FCB+DPPC suspension. The FCB suspended in BALF had no large agglomerates and possessed a more uniform dispersion pattern.

Comparison of $UFTiO_2$ and $FTiO_2$ suspensions in PBS, DPPC, and rat BALF

Upon examination of the 3.47 mg/ml $UFTiO_2$ suspensions, the $UFTiO_2$ suspended in PBS (Figure 3, panel A) had a uniform dispersion pattern of relatively small agglomerates.

The $UFTiO_2$ suspended in DPPC (Figure 3, panel B) had large agglomerates and did not exhibit a uniform dispersion pattern. The $UFTiO_2$ suspended in the BALF (Figure 3, panel C) had a uniform dispersion pattern with only small agglomerates. The $UFTiO_2$+PBS and the $UFTiO_2$+BALF had comparable dispersion patterns when viewed using light microscopy. However, when TEM was conducted on the same suspensions, the $UFTiO_2$ suspended in the BALF (Figure 4, panel B) had smaller agglomerates and a more uniform dispersion pattern than the $UFTiO_2$ suspended in the PBS (Figure 4, panel A).

Upon examination of the 8.93 mg/ml $FTiO_2$ suspensions, the $FTiO_2$ suspended in PBS (Figure 5, panel A) had large agglomerates and a non-uniform dispersion pattern.

The $FTiO_2$ suspended in DPPC (Figure 5, panel B) also had large agglomerates and a non-uniform dispersion pattern. In fact, the agglomerates in the $FTiO_2$+DPPC suspension were even larger than the agglomerates in the $FTiO_2$+PBS suspension. The $FTiO_2$ suspended in the BALF (Figure 5, panel C) had no large agglomerates and exhibited a uniform dispersion pattern. A SEM analysis of $FTiO_2$ suspended in PBS (Figure 6, panel A) also demonstrated larger agglomerates than the $FTiO_2$ suspended in the BALF (Figure 6, panel B).

Comparison of UFCB suspension in PBS, mouse BALF, and a combination of BSA and DPPC

After sonicating with 5 individual pulses, UFCB suspended in PBS had large agglomerates (Figure 7, panel A), whereas UFCB suspended in mouse BALF (Figure 7, panel B) or suspended in PBS containing 0.6 mg/ml BSA and 10 μg/ml DPPC (Figure 7, panel C) had noticeably smaller agglomerates.

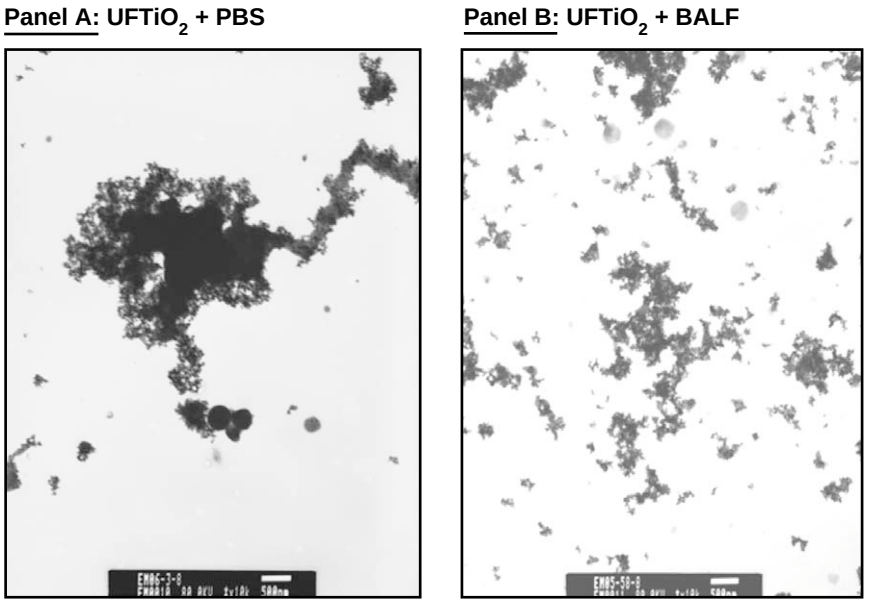

Figure 4. Transmission electron microscopy evidence for improved dispersion of ultrafine titanium dioxide ($UFTiO_2$) by bronchoalveolar lavage fluid (BALF). *Panel A*: 3.47 mg/ml of $UFTiO_2$ suspended in PBS. *Panel B*: 3.47 mg/ml of $UFTiO_2$ suspended in BALF. Samples were aerosolized as described in the methods and viewed with a transmission electron microscope. The $UFTiO_2$ suspended in the BALF (*panel B*) had noticeably smaller agglomerates and a more even and uniform dispersion pattern than the $UFTiO_2$ suspended in the PBS (*panel A*). TEM micrographs of $UFTiO_2$ suspensions were taken at a magnification of 30,000 ×.

UFCB suspended in BSA or DPPC alone exhibited noticeably more agglomeration than the BSA+DPPC vehicle (data not shown). The effect of sonication time on dispersion was also investigated. After a 10-min sonication, UFCB suspended in PBS had larger agglomerates (Figure 8, panel A) than UFCB suspended in PBS with a shorter sonication time (Figure 7, panel A).

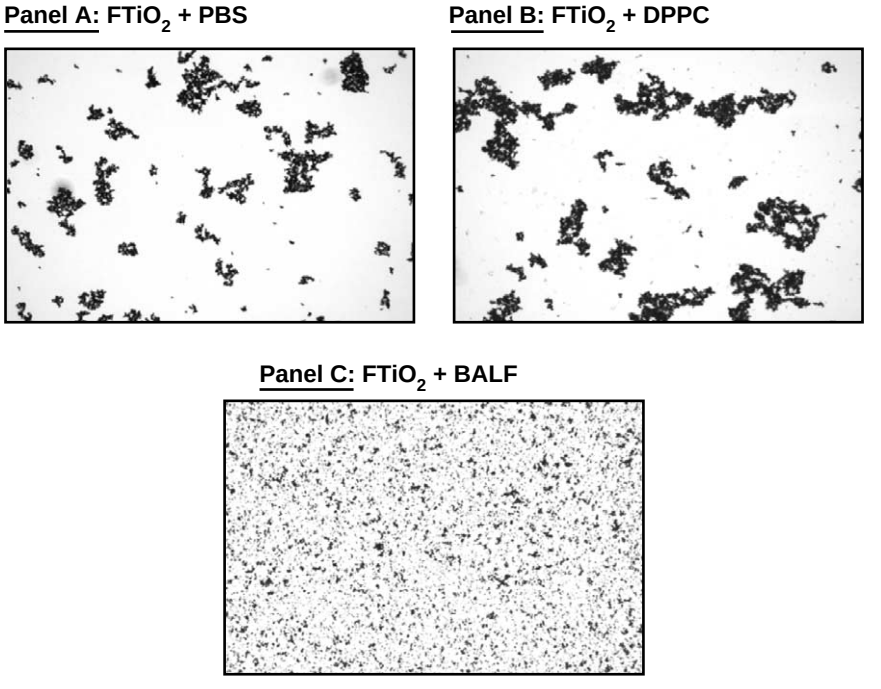

Figure 5. Light microscopy evidence for improved dispersion of fine titanium dioxide ($FTiO_2$) by bronchoalveolar lavage fluid (BALF). *Panel A*: 8.93 mg/ml $FTiO_2$ suspended in PBS. *Panel B*: 8.93 mg/ml $FTiO_2$ suspended in DPPC (160 μg/ml). *Panel C*: 8.93 mg/ml $FTiO_2$ suspended in BALF. Each particle suspension was sonicated with 5 individual pulses, and then a 25 μl sample was viewed using light microscopy at a magnification of 10 ×.

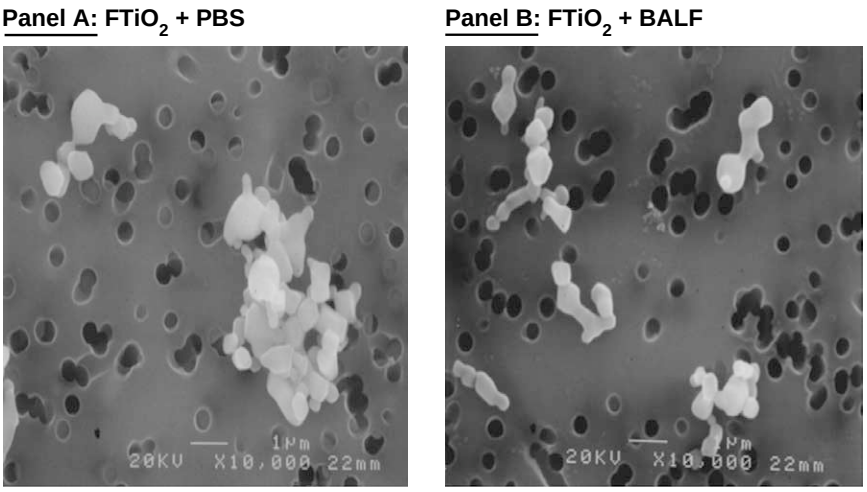

Figure 6. Scanning electron microscopy evidence of improved dispersion of fine titanium dioxide ($FTiO_2$) by bronchoalveolar lavage fluid (BALF). *Panel A*: 8.93 mg/ml FTiO2 suspended in PBS. *Panel B*: 8.93 mg/ml $FTiO_2$ suspended in BALF. Samples were aerosolized as described in the methods and viewed using scanning electron microscopy. The $FTiO_2$ suspended in PBS (*panel A*) had larger agglomerates than the $FTiO_2$ suspended in the BALF (*panel B*). SEM micrographs of $FTiO_2$ suspensions were taken at a magnification of 10,000 ×.

In contrast, when suspended in mouse BALF (Figure 8, panel B) or PBS containing 0.6 mg/ml BSA and 10 μg/ml DSPC (Figure 8, panel C), agglomerates decreased in size after a 10-min sonication compared to respective samples sonicated for the shorter duration (Figure 7, Panels B–C).

Comparison of toxicity of Min-U-Sil 2 silica in PBS and BALF

Thus far, data indicate that BALF noticeably improved the dispersability of ultrafine particles. To be a useful method to prepare particle suspensions for toxicity studies, there must be evidence that

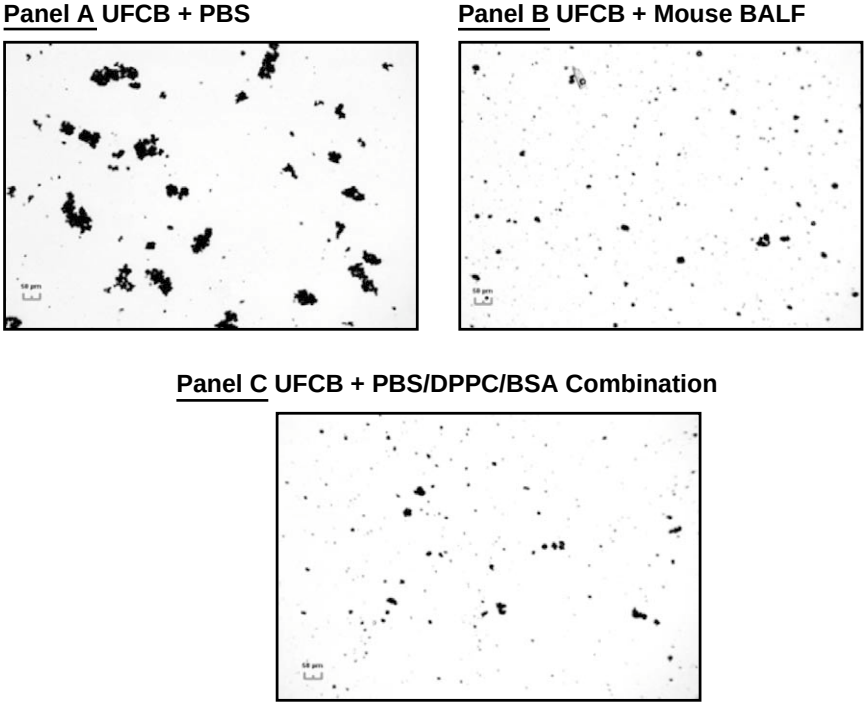

Figure 7. Light microscopy evidence of improved dispersion of ultrafine carbon black (UFCB) by a combination of bovine serum albumin (BSA) and dipalmitoyl phosphatidyl choline (DPPC). *Panel A*: UFCB (0.8 mg/ml) suspended in PBS. *Panel B*: UFCB (0.8 mg/ml) suspended in mouse BALF. *Panel C*: UFCB (0.8 mg/ml) suspended in PBS containing 0.6 mg/ml BSA and 10 μg/ml DPPC. Each particle suspension was sonicated with 5 individual pulses, and then a 25 μl sample was viewed using light microscopy at a magnification of 10 ×.

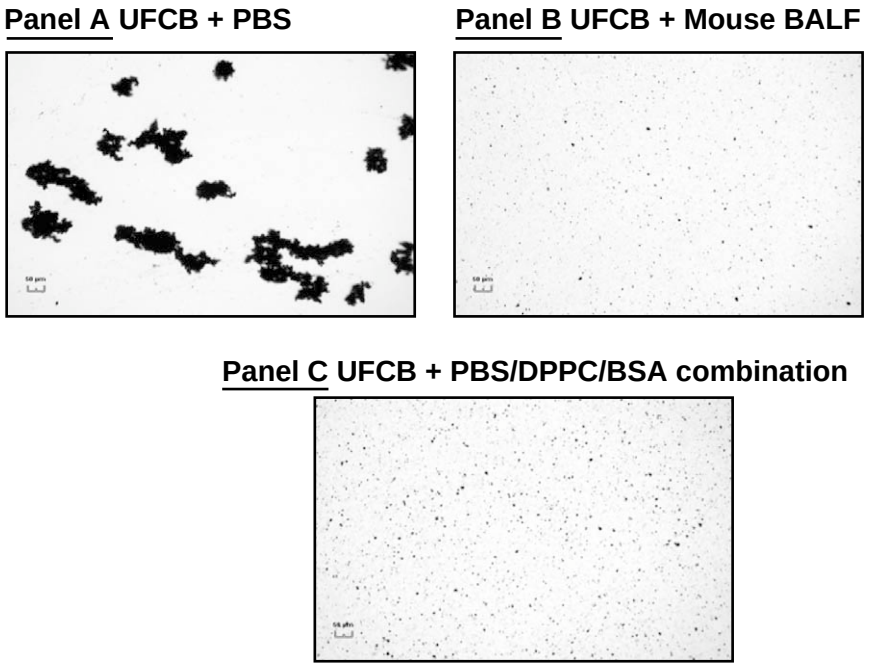

Figure 8. Light microscopy evidence of improved dispersion of ultrafine carbon black (UFCB) by a combination of bovine serum albumin (BSA) and dipalmitoyl phosphatidyl choline (DPPC), effect of sonication time. *Panel A*: UFCB (0.8 mg/ml) suspended in PBS. *Panel B*: UFCB (0.8 mg/ml) suspended in mouse BALF. *Panel C*: UFCB (0.8 mg/ml) suspended in PBS containing 0.6 mg/ml BSA and 10 μg/ml DPPC. Each particle suspension was sonicated for 10 min, and then a 25 μl sample was viewed using light microscopy at a magnification of 10 ×.

components in BALF fluid do not alter the bioactivity of the particle surface. We evaluated the pulmonary response to silica suspended in PBS or BALF after intratracheal instillation of quartz in rats. In comparison to PBS-exposed rats, exposure to silica suspended in PBS caused significant ($p < 0.05$) pulmonary inflammation as indicated by the large increase in the number of polymorphonuclear leukocytes (PMN) obtained by BAL (Figure 9).

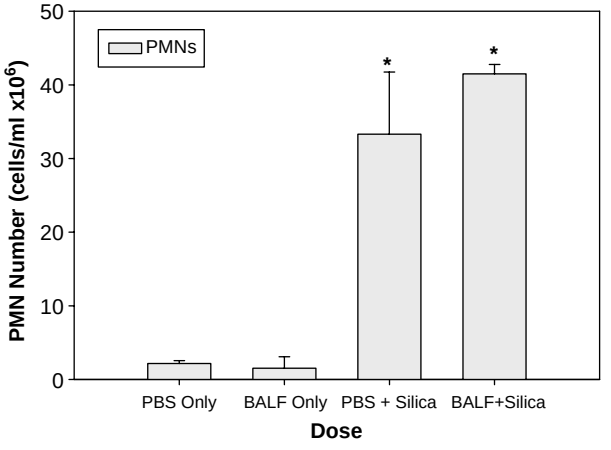

Figure 9. Suspension of silica in bronchoalveolar lavage fluid (BALF) does not affect *in vivo* inflammatory potential. PMN cell counts were determined in rats exposed to Min-U-Sil 2 silica (20 mg/rat) via intratracheal instillation (IT). The silica was dispersed in either PBS or BALF. Animals were euthanized 24 h post-IT, and bronchoalveolar lavage was performed. Silica suspended in BALF resulted in pulmonary inflammation that was similar to that seen with silica suspended in PBS. Experiments with animals instilled with PBS or BALF only showed that the BALF vehicle alone caused little inflammation with no significant difference between the two groups. Values are means ± SEM of 6 rats. *indicates a significant difference from the respective control ($p < 0.05$) using ANOVA.

In comparison to PBS vehicle-exposed rats BALF did not cause any inflammation and BALF did not alter the inflammatory potential of silica.

Instillation of silica suspended in PBS also caused significant ($p < 0.05$) pulmonary cytotoxicity as indicated by increased activity of LDH in the acellular BALF (Figure 10).

Instillation of BALF alone showed no toxicity, and suspension of silica in BALF did not alter the toxicity of silica.

Comparison of toxicity of Min-U-Sil 2 silica suspension in PBS, DPPC, and BALF

Exposure of A549 alveolar type II cells to silica resulted in a dose-dependent cytotoxicity as measured by LDH activity of the medium. There was no significant difference ($p > 0.05$) in the cytotoxicity of silica when suspended in PBS, DPPC or BALF, indicating that at the concentrations used, none of the components altered the surface activity of silica (Figure 11).

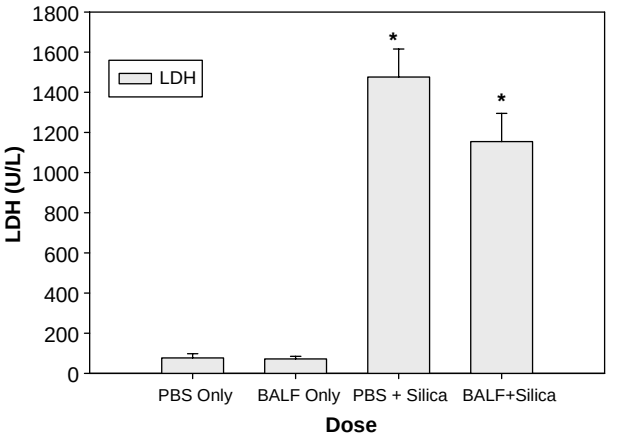

Figure 10. Suspension of silica in bronchoalveolar lavage fluid (BALF) does not affect *in vivo* cytotoxicity. Lactate dehydrogenase activity was measured in rats exposed to Min-U-Sil 2 silica (20 mg/rat) via intratracheal instillation (IT). The silica was dispersed in either PBS or BALF. Animals were euthanized 24 h post-IT and bronchoalveolar lavage was performed. Silica suspended in PBS or BALF both caused comparable cytotoxicity. There was no significant cytotoxicity in rats exposed to BALF alone compared to the PBS control. Values are means ± SEM of 6 rats. *indicates a significant elevation compared to the respective control ($p < 0.05$) using ANOVA.

Discussion

Nanoparticles tend to agglomerate into micrometer-sized structures in solution. Therefore, accurate dose delivery of nanosized particles becomes an important experimental issue. For testing the possible toxicity of nanoparticles, it is essential to disperse the nanomaterial as much as possible and characterize the physicochemical properties of the material as delivered to the test system (Oberdörster et al. 2005a).

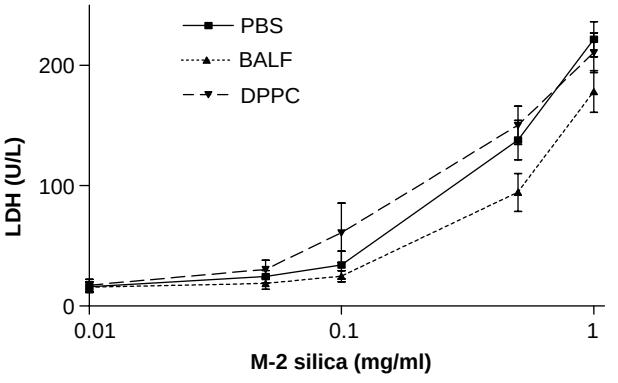

Figure 11. Suspension of silica in bronchoalveolar lavage fluid (BALF) does not affect *in vitro* cytotoxicity. Lactate dehydrogenase activity was measured in media removed from A549 cells cultured for 18 h with silica particles (<2 micrometers). The silica particles were suspended in either phosphate buffered saline (PBS), bronchoalveolar lavage fluid (BALF) or in PBS containing dipalmitoyl phosphatidylcholine (DPPC). Values are means ± SEM of 5 experiments. No significant differences in toxicity among the three silica suspensions were observed.

Data from the present study indicate that BALF (rat or mouse) is an excellent vehicle in which to suspend nanosized particles. Both mouse and rat BALF were examined in this study because both animal models are commonly used in *in vivo* toxicology studies. There is no significant difference between the two media. BALF from either rats or mice substantially decreased in the size and number of agglomerates compared to suspension in PBS. The use of protein alone or DPPC alone, in concentrations found in BALF, did not result in satisfactory dispersions. However, the combination of protein plus DPPC was a satisfactory, although slightly less effective, substitute for BALF.

When comparing Figures 1 through to 8 one can note the difference in the concentrations of fine versus ultrafine test particles that were used. It is of importance to address the reason as to why various concentrations of the particles being tested were utilized. It is known that the smaller the particle the greater the surface area, on an m^2/g basis. As the size of the particle decreases the surface area increases. The concentrations of the different sized particles used in this study were based on the particles surface area to mass ratio. The fine-sized particles used in the studies have a lower surface area-to-mass ratio because they have a larger primary particle size. This means that in order to closely match the doses of ultrafine and fine particles based on surface area, a higher mass concentration of fine particles must be used. For example, the ultrafine carbon black (Printex 90) used in our studies has a surface area of 269 m^2/g, while the fine carbon black (Aerosperse 15V) has a surface area of 8.1 m^2/g. Therefore, the large difference in surface areas of the two particle sizes means a large difference in the mass dose concentrations used.

The characterization of the physiochemical properties of the dispersion medium being delivered to the test system is another important experimental issue to consider. Previous studies have shown that DPPC pretreatment of quartz dust can fully suppress its cytotoxicity and apoptotic activity (Gao et al. 2001). This study brought about concern that the phospholipid present in our BALF dispersion medium might attenuate the toxicity of the particulates. However, it was found that BALF did not alter the inflammatory or toxic potential of silica *in vivo* or *in vitro*. This may be because the phospholipid levels of the BALF used as particle suspension medium (160 μg/ml) were significantly less than the levels of phopholipid (5 mg/ml) used by Gao et al. (2001). It was concluded that the level of phospholipid used in this current study is more physiologically relevant and does not coat the particle in such a way as to mask its biological activity.

An inquiry can be made as to why our study utilized silica rather than nanoparticles in the *in vivo*

studies assessing the particle coating issues. Fine-sized silica was used as a 'model' or 'surrogate' particle in the *in vivo* studies because it is well dispersed in PBS. The doses of silica used in the *in vivo* studies were based on previous lab experiments which assessed silica toxicity. From our lab data, the dose chosen would induce significant lung damage and inflammation but was not the maximum dose of silica for 24-h post responses. Fine sized silica, instead of a nanoparticle, was utilized in the *in vivo* studies in order to examine whether or not the DPPC coating attenuated the toxicity of the particle. If nanoparticles, were used one would have had two independent variables, i.e., particle dispersion and particle coating. As nanoparticle dispersion goes up, one would predict toxicity to increase. If nanoparticle coating occurs, one would expect toxicity to decrease. Thus, if nanoparticles were used, it would be possible that these two effects could cancel out. By using fine-sized silica, we eliminated the particle dispersion variable, and can solely address the 'particle coating' question, which increased in significance after Gao et al. (2001) reported that fully coating silica with DPPC suppressed its cytotoxicity by masking surface active groups on silica particles.

Another important issue can be raised as to whether improved dispersion of nanoparticles makes a significant difference in biological reactivity. We have conducted *in vivo* studies to directly address this issue (Shvedova et al. 2007). In this study, rats were exposed to two different prepartions of ultrafine carbon black (Printex 90, Degussa Corporation, Parsippany, NJ; 14 nm primary particle diameter), i.e., one where particles were suspended in phosphate-buffered saline and the other where particles were suspended in BALF. The suspensions were sonicated as described in the present paper, and rats were exposed by intratracheal instillation. At 24 h post-exposure, dose-dependent pulmonary damage and inflammation were determined by measuring BALF LDH or the number of lavagable polymorphonuclear leukocytes (PMNs), respectively. Instillation of BALF fluid alone had no effect. However, ultrafine carbon black dispersed in BALF caused a 1.7-fold greater cytotoxicity and 2-fold greater inflammation than the poorly dispersed ultrafine carbon black at a maximum concentration (1.5 mg/rat). Also, an equivalent pulmonary response was seen at a 16-fold lower dose of BALF-dispersed carbon black compared to carbon black dispersed in phosphate-buffered saline. In addition, unpublished data from our laboratory indicate that improved dispersion of single-walled carbon nanotubes resulted in significantly enhanced potency of aspirated material to induce interstitial fibrosis in mice.

Another indication of the importance of nanoparticle dispersal in subsequent biological response is a study by Warheit et al. (2007). In this study, two ultrafine TiO_2 (rutile) samples (surface areas of 18.2 and 35.7 m^2/g, respectively) were compared with a fine TiO_2 (rutile) sample (surface areas of 5.8 m^2/g). Although the ultrafine particles had a much smaller primary particle diameter than the fine TiO_2, upon suspension in saline, all three TiO_2 samples exhibited agglomerated particle diameters of approximately 2.6 μm. Intratracheal instillation of these poorly dispersed ultrafine and fine TiO_2 preparations resulted in a similar level of inflammation, cytotoxicity, cell proliferation and histopathological response. One might conclude that particle surface area (measured by gas absorption of dry material) was not a factor in biological potency. However, the results may reflect that the physical surface area of the agglomerated particles as delivered to the rats did not differ. Therefore, it is critical to make an effort to disperse nanoparticles before evaluating toxicity in *in vitro* cultures or with *in vivo* animal models exposed to nanomaterials in suspension, i.e., via pharyngeal aspiration or intratracheal instillation.

In conclusion, several important observations were made in this study. First, PBS is not a satisfactory medium to prepare particle suspensions of nanosized particles. The formation of aggregates when using PBS as a particle suspension medium hinders the accuracy of dose estimations delivered to the test system. This inaccurate dose delivery leads to misinterpretation of the toxicity of the particulate being assessed. Second, suspension of nanoparticles in acellular BALF is effective in dispersing the nanoparticles without masking the biological activity of the surface. BALF as a suspension medium significantly reduces agglomeration of nanoparticles in solution. This in turn increases the accuracy of the dose being delivered to the testing system. Evidence indicates that a mixture of protein and DPPC at levels found in BALF is an adequate, although not quite equivalent, substitution for BALF.

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.

References

Donaldson K, Aitken R, Tran L, Stone V, Duffin R, Forrest G, Alexander A. 2006. Carbon nanotubes: A review of their properties in relation to pulmonary toxicology and workplace safety. Toxicolog Sci 92(1):5–22.

Donaldson K, Brown D, Clouter A, Duffin R, MacNee W, Renwick L, Tran L, Stone V. 2002. The pulmonary toxicology of ultrafine particles. J Aerosol Med 15(2):213–220.

Donaldson K, Stone V, Clouter A, Renwick L, MacNee W. 2001. Ultrafine particles. Occupat Environ Med 58:211–216.

Gao N, Keane M, Ong T, Ye J, Miller W, Wallace W. 2001. Effects of phospholipid surfactant on apoptosis induction by respirable quartz and kaolin in NR8383 rat pulmonary macrophages. Toxicol Appl Pharmacol 175:217–225.

Nel A, Xia T, Madler L, Li N. 2006. Toxic potential of materials at the nanolevel. Science 311:622–627.

Oberdörster 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, Yang H. 2005a. Principles for characterizing the potential human effects from exposure to nanomaterials: Elements of a screening strategy. Particle Fibre Toxicol 2:8.

Oberdörster G, Oberdörster E, Oberdörster J. 2005b. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 133(7):823–839.

Porter DW, Ramsey D, Hubbs AF, Battelli L, Ma J, Barger M, Landsittel D, Robinson VA, McLaurin J, Khan A, Jones W, Teass A, Castranova V. 2001. Time course of pulmunary response of rats to inhalation of crystalline silica: histological results and biochemical indices of damage, lipidosis and fibrosis. J. Environ. Pathol. Toxicol. Oncol. 20(Suppl 1): 1–14.

Porter D, Barger M, Robinson V, Leonard S, Landsittel D, Castranova V. 2002. Comparison of low doses of aged and freshly fractured silica on pulmonary inflammation and damage in the rat. Toxicology 175:63–71.

Seaton A, Donaldson K. 2005. Nanoscience, nanotoxicology, and the need to think small. The Lancet 365:923–924.

Shvedova A, Kisin E, Mercer R, Murray A, Johnson V, Potapovich A, Tyurina Y, Gorelik O, Arepalli S, Schwegler-Berry D, Hubbs A, Antonini J, Evans D, Ku B, Ramsey D, Maynard A, Kagar V, Castranova V, Baron P. 2005. Unusual inflammatory and fibrogenic response to single walled carbon nanotubes in mice. Am J Physiol: Lung Cell Molec Physiol 289:L698–708.

Shvedova A, Sager T, Murray A, Kisin E, Porter D, Leonard S, Schwegler-Berry D, Robinson V, Catranova V. 2007. Critical issues in the evaluation of possible adverse pulmonary effects resulting from airborne nanoparticles. In: Monteiro-Rivieren, Tranl, editors. Nantechnology: Characterization, dosing and health effects. New York: Taylor and Francis.

Warheit DB, Laurence BR, Reed KL, Roach DH, Reynolds GAM, Webb TR. 2004. Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rat. Toxicol. Sci. 77:117–125.

Warheit D, Webb T, Reed K, Frerichs S, Sayes C. 2007. Pulmonary toxicity study in rats with three forms of ultrafine TiO2 particles: Differential responses related to surface properties. Toxicology 230(1):90–104.

Zeidler P, Hubbs A, Battelli L, Castranova V. 2004. Role of nitric oxide synthase-derived nitric oxide in silica-induced pulmonary inflammation and fibrosis. J Toxicol Environ Health A 67:1001–1026.