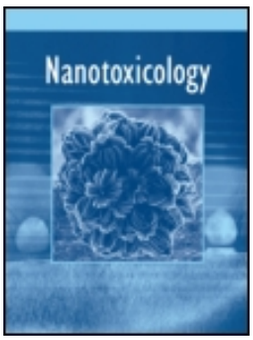

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A biocompatible medium for nanoparticle dispersion

DALE PORTER[1,2], KRISHNAN SRIRAM[1], MICHAEL WOLFARTH[1], AMY JEFFERSON[1], DIANE SCHWEGLER-BERRY[1], MICHAEL ANDREW[1], & VINCENT CASTRANOVA[1,2]

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

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Abstract
Our laboratory has reported that rat bronchoalveolar lavage (BAL) fluid is an effective nanoparticle (NP) dispersant. However, its utility is constrained by its cost and the lack of standardization to control for intra- and inter-laboratory variability in BAL fluid. In this study, we report the efficacy and biocompatibility of a dispersion medium (DM), which is a 'lung fluid mimic'. *In vitro* studies, which used dynamic light scattering and transmission electron microscopy, determined that ultrafine titanium dioxide and ultrafine carbon black are equally well dispersed by DM or BAL fluid. We also determined that DM was effective at dispersing multi-walled carbon nanotubes. *In vivo*, when used as a vehicle, DM per se did not elicit toxicity and did not influence or alter toxic responses to crystalline silica in either the lung or brain. Overall, these studies indicate that DM is an effective, biocompatible, and economical vehicle for nanotoxicological studies.

Keywords: *Nanoparticles, nanotoxicology, nanotubes*

Introduction

With the rapid development of nanotechnology, engineered nanoparticles are receiving intense interest because of their potential applications in the production of consumer goods and medical products. Workers are at risk for exposure to nanoparticles during manufacturing, handling, and cleanup processes (Colvin 2003; Dreher 2004; Maynard & Kuempel 2005). In order to predict and reduce the risk of occupational illness associated with nanoparticles, it is necessary to study their potential toxicological effects. Investigations into the risk of occupational illness associated with nanoparticles are just beginning, and it is being realized that nanosized particles present distinct challenges regarding the study of their potential toxicological effects.

Besides size, one distinctive property of nanoparticles is their tendency to agglomerate. This is a term used to describe a collection of primary nanoparticles held together by a combination of weak and/or strong forces, e.g., van der Waals forces, electrostatic forces, and sintered bonds. The degree and type of agglomerate formed are thought to influence their toxicity (Sager et al. 2007; Shvedova et al. 2007; Wick et al. 2007). Nanoparticle agglomeration represents a major problem associated with the conduct and interpretation of nanoparticle toxicity studies, and the development of an appropriate nanoparticle dispersion medium is needed for such studies.

Differing strategies have been used by a variety of laboratories to disperse nanoparticles for toxicology studies. For example, *in vitro* studies have used cell culture medium (Shvedova et al. 2003; Jia et al. 2005), while *in vivo* studies have used 1% Tween 80 in phosphate buffered saline (Warheit et al. 2004; Muller et al. 2005). More recently, our laboratory has reported the use of rat bronchoalveolar lavage (BAL) fluid as a nanoparticle dispersion medium for *in vitro* and *in vivo* studies (Sager et al. 2007). Other studies have independently demonstrated that addition of proteins and surfactants may facilitate nanoparticle dispersion. Serum albumin, by virtue of its amphoteric nature, binds to nanoparticles and stabilizes them (Singh et al. 2005), whereas surfactants decreased the agglomeration of nanoparticles (Vaisman et al. 2007). The presence of protein and pulmonary surfactants in BAL fluid is likely the basis for the dispersive effects of BAL fluid on nanoparticles in our previous study.

Although BAL fluid is an effective dispersion medium, its utility is constrained by several factors. First, the economics of preparing BAL fluid potentially limits its use. To obtain BAL fluid, a separate

Correspondence: Dale Porter, PhD, National Institute for Occupational Safety and Health, 1095 Willowdale Road, M/S 2015, Morgantown, WV, 26505, USA. E-mail: dporter@cdc.gov

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set of naïve animals must be used, and generating BAL fluid requires a significant investment in time from investigators. Combined, both of these factors dramatically increase the cost associated with using BAL fluid as a nanoparticle dispersion solution. Secondly, BAL fluid must be derived from the specific species in which experimental procedures are to be conducted in order to avert any possible adverse cross-species reactions. If a mouse model is to be used, this could prove to be a major limitation due to the low volume of BAL fluid that can be obtained. Third, intra- and inter-laboratory variability in BAL fluid is a problem due to differences in many factors, including animal handling, anesthesia, and BAL technique, as well as the inherent complex composition of BAL fluid.

Another major concern with BAL fluid, as well as similar commercially available animal derived products (e.g., Survanta®, Curosurf®), is that they contain surfactant proteins. Surfactant protein-A (SP-A) has been shown to inhibit mRNA expression of TNF-α, IL-1α and IL-β (Alcorn & Wright 2004), and to increase apoptotic polymorphonuclear leukocytes (PMNs) uptake and TGF-β1 release by alveolar macrophages (AMs) (Reidy & Wright 2003). SP-A also prevents silica-mediated toxicity of alveolar macrophages (Spech et al. 2000). Surfactant protein-B (SP-B) has been shown to inhibit LPS-induced nitric oxide (NO) production by AMs via reduction in inducible nitric oxide synthase (iNOS) protein level (Miles et al. 1999). *In vitro*, Survanta® has been shown to inhibit the phagocytosis of Streptococcus pneumoniae by rat AMs, indicating a suppression of host defense function (Golioto & Wright 2002). These findings indicate that surfactant proteins which are present in BAL fluid, as well as other commercially available animal derived products may alter AM responses to nanoparticles. This possibility makes them unsuitable as vehicles for nanoparticle exposures, since they may alter AM function in the absence of a nanoparticle.

Because of these factors, our laboratory initiated studies to develop an alternative nanoparticle dispersion medium to BAL fluid and other similar commercially available animal derived products, e.g., Survanta® and Curosurf®. Based on the success of our laboratory using BAL fluid as a nanoparticle dispersant, and studies by other laboratories that have indicated protein and surfactant are important components of a dispersion solution, we developed a BAL fluid mimic, i.e., Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS) containing serum albumin and a lung surfactant, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). We call this preparation dispersion medium (DM). It is important to note that DM mimics components of the lung alveolar lining fluid important to nanoparticle dispersion, i.e., protein and surfactant, but at concentrations much lower than those present in lung alveolar lining fluid.

In order to evaluate DM for use in nanotoxicology studies, we conducted: (i) *In vitro* investigations which compared nanoparticle dispersion in PBS, BAL fluid and DM, and (ii) an *in vivo* investigation to determine whether DM causes toxicity in the pulmonary or central nervous system when administered to mice by pharyngeal aspiration.

Methods

Animals

All animals used in this study were housed in an AAALAC-accredited, specific pathogen-free, environmentally controlled facility. Male C57BL/6J mice (7 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME), and male Sprague-Dawley [Hla®(SD)CVF®] rats weighing 200–225 g were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). Animals were housed one per cage in polycarbonate isolator ventilated cages, which were provided HEPA-filtered air, with fluorescent lighting from 07:00 to 19:00 h. Autoclaved Alpha-Dri virgin cellulose chips and hardwood Beta-chips were used as bedding. Animals were monitored to be free of endogenous viral pathogens, parasites, mycoplasms, Helicobacter and CAR Bacillus. Mice were maintained on Harlan Teklad Rodent Diet 7913 (Indianapolis, IN) and rats were maintained on Harlan Teklad Rodent Diet 2918 (Indianapolis, IN); tap water was provided *ad libitum*. Animals were allowed to acclimate for at least five days before use.

Particles

Fine silica (Min-U-Sil 5, U.S. Silica, Berkley Springs, WV; lot #15121996), ultrafine titanium dioxide (UFTiO$_2$; Degussa Aeroxide P25, lot #PIS18C1), ultrafine carbon black (UFCB; Degussa Printex 90, lot #0234121), and multi-walled carbon nanotubes (MWCNT; Mitsui & Company, XNRI MWNT-7, lot #05072001K28) were used in these studies.

Dispersion medium (DM)

Dispersion medium (DM) is Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS), pH 7.4, supplemented with 5.5 mM D-glucose, 0.6 mg/ml species-specific serum albumin, and 0.01 mg/ml 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). DPPC was prepared fresh as a 10 mg/ml stock solution in absolute ethanol (200 proof). For dynamic

light scattering studies, DM solutions were prepared using bovine serum albumin (BSA), whereas for *in vivo* studies mouse serum albumin was used. DM was briefly sonicated (Branson Sonifier 450, 10 W continuous output, 1 minute) before use.

Rat bronchoalveolar lavage (BAL) fluid isolation

Naïve rats were euthanized with an i.p. injection of sodium pentobarbital (>100 mg/kg body weight) followed by exsanguination. Next, a tracheal cannula was inserted and bronchoalveolar lavage (BAL) was performed through the cannula using 6 ml of ice cold PBS. BAL cells were isolated by centrifugation (650 *g*, 5 min, 4°C) and the acellular supernatant (BAL fluid) thus obtained was used for *in vitro* particle sizing studies.

Dynamic light scattering studies

Suspensions of UFTiO$_2$ and UFCB, each at 0.5 mg/ml, were prepared in PBS, rat BAL fluid, or DM. Particle size was determined by dynamic light scattering using Nanotrac 252 (Microtrac, Montgomeryville, PA) immediately after vortexing, and at 0 and 24 h following 30 min of sonication with a probe sonicator (Branson Sonifier 450, 10 W continuous output). During sonication, heat was dissipated by placing the samples on ice.

MWCNT Dispersion

Suspensions of MWCNT (1.8 mg/ml) were prepared in PBS or DM. A two-step sonication process was used to disperse the MWCNT. First, the samples underwent indirect sonication (Hielscher ultrasonic processor UIS250v with vial tweeter, 10 W continuous output) for 5 min at 4°C. Next, the samples were sonicated with a probe sonicator (Branson Sonifier 450, 5 W output, 10% duty cycle) for 5 min at room temperature. This two-step sonication process was used because we empirically determined that if MWCNTs were probe sonicated immediately after initial vortexing, much of the MWCNT adhered to the sonicator probe. However, when indirect sonication was performed prior to probe sonication, MWCNT adherence to the probe sonicator did not occur.

Mouse pharyngeal aspiration

Mice were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL, USA). When fully anesthetized, the mouse was positioned with its back against a slant board and suspended by the incisor teeth using a rubber band. The mouth was opened, and the tongue gently pulled aside from the oral cavity. A 50 μl aliquot of sample was pipetted at the base of the tongue, and the tongue was restrained until at least 2 deep breaths were completed (but for not longer than 15 sec). Mice received either: (i) PBS, (ii) DM, (iii) 1 mg silica suspended in PBS (PBS/Si), or (iv) 1 mg silica suspended in DM (DM/Si). Following release of the tongue, the mouse was gently lifted off the board, placed on its left side, and monitored for recovery from anesthesia.

Mouse bronchoalveolar lavage

At one day post-exposure, mice were euthanized with an i.p. injection of sodium pentobarbital (>100 mg/kg body weight) followed by exsanguination. A tracheal cannula was inserted and bronchoalveolar lavage (BAL) was performed through the cannula using ice cold PBS. The first lavage (0.6 ml) was kept separate from the rest of the lavage fluid. Subsequent lavages, each with 1 ml of PBS, were performed until a total of 4 ml of lavage fluid was collected. BAL cells were isolated by centrifugation (650 *g*, 5 min, 4°C). An aliquot of the acellular supernatant from the first BAL (BAL fluid) was decanted and transferred to tubes for analysis of lactate dehydrogenase (LDH) and albumin. The acellular supernatants from the remaining lavage samples were decanted and discarded. BAL cells isolated from the first and subsequent lavages for the same mouse were pooled after resuspension in PBS, centrifuged a second time (650 *g*, 5 min, 4°C), and the supernatant decanted and discarded. The BAL cell pellet was then resuspended in PBS and placed on ice.

BAL cell differentials

Total BAL cell counts were obtained using a Coulter Multisizer 3 (Coulter Electronics, Hialeah, FL) and cytospin preparations of the BAL cells were made using a cytocentrifuge (Shandon Elliot Cytocentrifuge, London, UK). The cytospin preparations were stained with modified Wright-Giemsa stain. As previously described by our laboratory (Porter et al. 2002), differential cell counts were calculated by multiplying the total cell counts obtained from the Coulter Multisizer 3 by the cell differential percentages obtained from the cytospin preparations.

BAL fluid lactate dehydrogenase

BAL fluid LDH activities were evaluated as a marker of cytotoxicity. BAL fluid LDH activities were determined by monitoring the LDH catalyzed oxidation of lactate to pyruvate coupled with the reduction of NAD^+ at 340 nm using a commercial assay kit (Roche Diagnostics Systems, Montclair,

NJ, USA). BAL fluid LDH assays were conducted using a COBAS MIRA Plus (Roche Diagnostic Systems, Montclair, NJ, USA).

RNA isolation, cDNA synthesis, and real-time PCR

A separate set of animals was treated for PCR studies. Mice were euthanized with an i.p. injection of sodium pentobarbital (>100 mg/kg body weight) followed by exsanguination. Immediately after euthanasia, the lung and brain areas (olfactory bulb, hippocampus, frontal cortex) were removed/dissected and placed in RNA*Later*® (Ambion, Austin, TX, USA). The tissues were homogenized in Tri Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) and the aqueous phase separated with MaXtract High Density gel (Qiagen, Valencia, CA, USA). Total RNA from the aqueous phase was then isolated using RNeasy mini spin columns (Qiagen, Valencia, CA, USA) and concentrations determined with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

First strand cDNA synthesis was carried out using total RNA (1 μg), random hexamers and MultiScribe™ reverse transcriptase (High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a 20 μl reaction.

Real-time PCR amplification was performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in combination with TaqMan® chemistry. Specific primers and FAM™ dye-labeled TaqMan® MGB probe sets (TaqMan® Gene Expression Assays) for CCL2 (MCP1; Assay ID Mm00441242_m1), CXCL2 (MIP2; Assay ID Mm00436450_m1), TNFα (Assay ID Mm00443258_m1), PTGS2 (COX2; Assay ID Mm00478374_m1), HMOX1 (HO1; Assay ID Mm00516004_m1), and endogenous control GAPDH (Part number 4352932E) were procured from Applied Biosystems (Foster City, CA, USA) and used according to the manufacturer's recommendation. All PCR amplifications (40 cycles) were performed in a total volume of 50 μl, containing 1 μl cDNA, 2.5 μl of the specific TaqMan® Gene Expression Assay, and 25 μl of TaqMan® Universal master mix (Applied Biosystems, Foster City, CA, USA), respectively. Sequence detection software (version 1.7; Applied Biosystems, Foster City, CA, USA) results were exported as tab-delimited text files and imported into Microsoft Excel for further analysis. Relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer (Applied Biosystems, Foster City, CA, USA; User Bulletin 2). Relative changes in mRNA expression were calculated following normalization to GAPDH.

Transmission electron microscopy

Nanoparticle samples were prepared as described (see Dynamic Light Scattering Studies and MWCNT Dispersion sections above) in either PBS, BAL fluid, or DM, then diluted 1:1000 with dH_2O. Without this dilution step, salt and protein components of the various solutions used to disperse nanoparticles created significant artifacts in the micrographs that prevented their interpretation. A drop (approximately 0.1 ml) was deposited onto a formvar-coated copper grid and allowed to air dry. Images were photographed on a JEOL 1220 transmission electron microscope.

Statistical analyses

Since variances between treatment groups were quite different, all statistical tests for group differences were performed using SAS Proc Mixed with an option that allows for unequal variance between experimental groups. Specific group comparisons were performed using the least squares means post-hoc *t* tests available in Proc Mixed. Since the effects of sonication on particle size (Table I) had a repeated measures design over three time points, these data were modeled, and the covariance between time measurements accounted for, using Proc Mixed with an unstructured covariance structure. This approach allowed for different variances at each time point.

Results

Dispersion of compact nanoparticles

Dynamic light scattering (DLS) measurements were made *in vitro* to examine the dispersion of $UFTiO_2$ and UFCB in three different solutions: PBS, BAL fluid and DM (Table I). BAL fluid was obtained from rats, since our laboratory has previously reported the use of rat BAL fluid as a nanoparticle dispersion medium (Sager et al. 2007). After vortexing, $UFTiO_2$ existed as large agglomerates in all three solutions, ranging in size from 863–2,381 nm. Sonication of $UFTiO_2$ in PBS resulted in an increase in mean particle size from 1,930 nm (after vortexing) to 2,849 nm, immediately post-sonication (0 hour). By 24 h post-sonication, the particle size increased further, indicative of greater agglomeration. In contrast, sonication of $UFTiO_2$ in BAL fluid or DM significantly decreased particle size by 76.4% and 93.2%, respectively, as compared to values after vortexing. Specifically, mean $UFTiO_2$ particle sizes in BAL fluid and DM were 204 nm and 163 nm at

Table I. Effect of carrier fluid and sonication on nanoparticle size.

		Particle size (nm)		
			Post-sonication (hours)	
Nanoparticle	Carrier fluid	After vortexing	0	24
$UFTiO_2$	PBS	$1{,}930 \pm 197^{a,1}$	$2{,}849 \pm 385^{a,b,1}$	$3{,}094 \pm 290^{b,1}$
	BAL fluid	$863 \pm 78^{a,2}$	$204 \pm 18^{b,2}$	$499 \pm 134^{a,c,2}$
	DM	$2{,}381 \pm 236^{a,1}$	$163 \pm 5^{b,2,3}$	$184 \pm 3^{c,3}$
UFCB	PBS	N.D.	N.D.	N.D.
	BAL fluid	N.D.	$131 \pm 4^{a,1}$	$149 \pm 2^{b,1}$
	DM	N.D.	$93 \pm 3^{a,2}$	$144 \pm 5^{b,1}$

For each nanoparticle, values within the same column with different numerical superscripts are significantly different; For each nanoparticle, values within the same row with different alphabetical superscripts are significantly different; N.D., not determinable.

0 h post-sonication. The particle sizes increased marginally 24 h post-sonication in both BAL fluid and DM relative to 0 hour values, but were still significantly smaller compared to similar samples in PBS. These DLS observations suggest that $UFTiO_2$ particles appear to be better dispersed in BAL or DM fluids. In order to corroborate the DLS measurements, transmission electron microscopy (TEM) examination of $UFTiO_2$ preparations were performed (Figure 1). These TEM micrographs reflected particle size characteristics consistent with those determined by DLS.

DLS measurements of UFCB particle size were not determinable when suspended and vortexed in PBS, BAL fluid, or DM (Table I). Inspection of these solutions indicated large agglomerates, easily discernable to the naked eye. The presence of such large agglomerates interfered with particle size measurements by DLS, as these measures are based, in part, on random fluctuations arising from Brownian motion of small particles. Indeed, the agglomerates were so large they sedimented by gravity within a few seconds after vortexing was complete. However, upon sonication in the presence of BAL or DM fluids, but not PBS, measurements were possible due to reductions in particle size. At 0 hours post-sonication, mean UFCB particle sizes in BAL fluid and DM were 131 and 93 nm, respectively. Twenty-four hours after sonication, UFCB particle sizes in both BAL fluid and DM increased slightly relative to those at 0 hours post-sonication.

MWCNT dispersion

DLS provides diffusion equivalent diameter of a particle. MWCNTs have a fiber-like morphology, which makes DLS measurements of MWCNT difficult to interpret. Thus, we used TEM to qualitatively assess their dispersion in PBS and DM. Initial visual examination of MWCNT in PBS showed the nanotubes were highly agglomerated into large masses (Figure 2, panel A), whereas in DM the MWCNT appeared to be more dispersed (Figure 2, panel C). This conclusion was supported by TEM micrographs, which showed MWCNT suspended in PBS (Figure 2, panel B) were highly agglomerated. In comparison, MWCNT suspended in DM were much better dispersed (Figure 2, panel D). It should be noted that due to the presence of the large agglomerates in MWCNT suspended in PBS, only small areas with highly dense MWCNT agglomerates were observed in the TEM sample, with the majority of the sample devoid of any MWCNT. However, in the MWCNT dispersed in DM, the MWCNT were more evenly distributed throughout the TEM sample, reflecting their dispersion status relative to that in PBS.

Utility of DM for toxicological studies: Lung and brain responses

Having demonstrated in the DLS studies that nanomaterials are better dispersed in DM, an important question that remained to be addressed was the suitability of the DM for toxicological studies. Two issues were addressed: DM biocompatibility (i.e., does DM cause toxicity per se), and effect of DM on the bioactivity of particles (i.e., does DM mask the particle surface). We investigated these effects by monitoring the inflammatory potential of DM alone, as well as the effect of DM on the inflammatory potency of crystalline silica.

Pulmonary inflammation and damage were assessed one day after exposure to either PBS, DM, PBS/Si, or DM/Si. No significant difference in PMNs or LDH was determined between PBS- and DM-exposed mice (Figure 3), indicating that DM does not cause any pulmonary inflammation or cytotoxicity when used as a vehicle. Comparison of responses to PBS/Si versus DM/Si exposure groups was mixed. BAL PMNs after exposure to DM/Si were significantly higher than those in the PBS/Si group, whereas BAL fluid LDH values did not differ significantly.

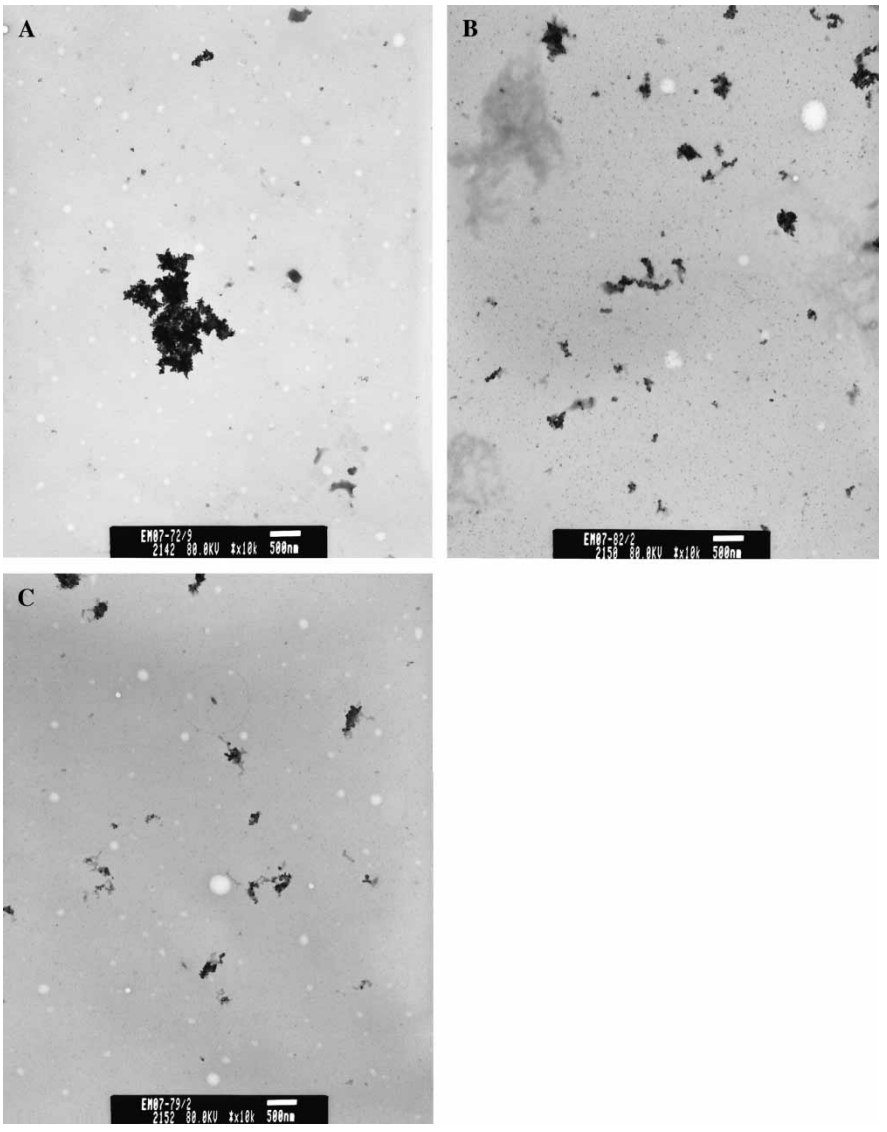

Figure 1. Transmission electron microscopy (TEM) micrographs of UFTiO$_2$ in PBS (A), rat BAL fluid (B) and DM (C) immediately after 30 minutes' sonication. Samples for TEM analyses were obtained from the same samples used for dynamic light scattering analyses at 0 hours post-sonication (data presented in Table I).

Lungs were also analyzed at 1 day post-exposure by real-time PCR to assess the expression of mRNAs for inflammatory cytokines and enzymes related to oxidative stress. No significant differences in the mRNA expression of lung cytokines (Figure 4) or oxidative stress markers (Figure 5) were observed in PBS- or DM-exposed mice, indicating that exposure to DM does not induce any change in the expression of these inflammatory or oxidative stress markers. Responses to PBS/Si versus DM/Si exposure groups were also similar in that the elevations in inflammatory cytokines (Figure 5) or oxidative stress genes (Figure 6) were not statistically different.

We also analyzed the central nervous system (CNS) effects of pulmonary exposure to crystalline silica 1 day post-exposure. Consistent with the pulmonary studies above, PBS or DM alone did not induce neuroinflammatory responses, as assessed by the induction of TNFα mRNA (Figure 6). Silica exposure elicited a 2.5- to 4-fold ($p < 0.05$) increase in TNFα mRNA expression in various brain areas (olfactory bulb, hippocampus, frontal cortex) one day after pulmonary exposure. However, no significant difference in the neurotoxic responses was observed between the two preparations of silica, PBS/Si or DM/Si, indicating that DM does not alter particle toxicity.

Discussion

Dispersion of nanomaterials is an important criterion for the accurate evaluation of toxicological responses when exposure requires suspension of nanoparticles in a liquid vehicle. Here, we have

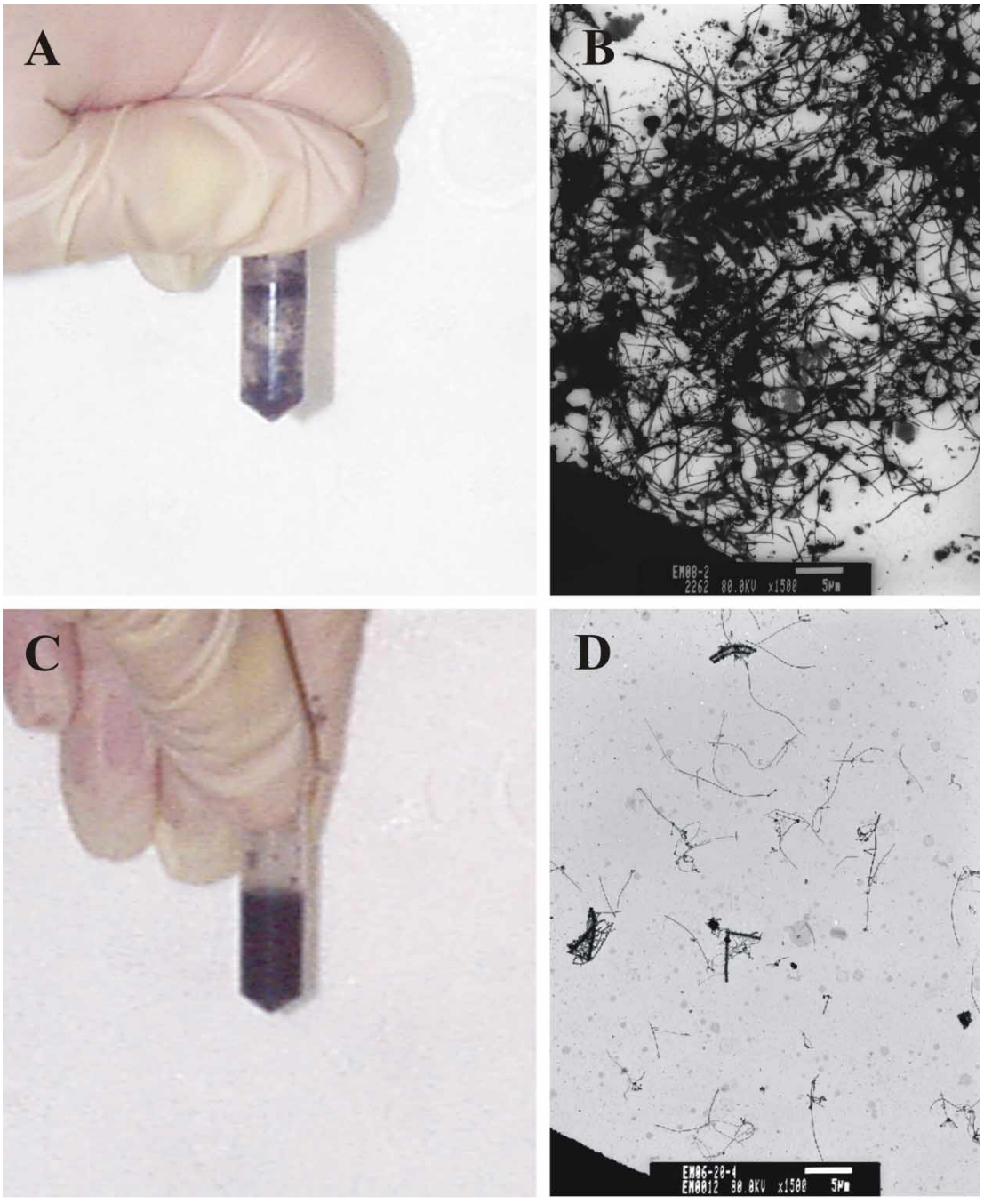

Figure 2. Dispersion of MWCNT in PBS and DM. Pictures and TEM micrographs of MWCNT suspended in PBS (A and B) or DM (C and D).

demonstrated the efficacy and biocompatibility of a 'lung fluid mimic' in dispersing nanomaterials. The DM developed is an artificial physiological buffer comprised of protein and surfactant components naturally found in lung alveolar fluids.

To evaluate DM for use in nanotoxicology studies, we initially conducted *in vitro* investigations which compared nanoparticle dispersion in PBS, BAL fluid, and DM. Sonication for specific periods of time was essential for dispersing $UFTiO_2$ and UFCB in both rat BAL fluid and DM, which resulted in a significant reduction in particle size relative to vortexing. Furthermore, after sonication, $UFTiO_2$ and UFCB particle sizes were similar in BAL fluid and DM, respectively, and were significantly smaller than suspensions in PBS, indicating that DM was an equally effective dispersant as BAL fluid. These experiments indicated that DM is as effective as BAL fluid in dispersing metal oxide and carbon-based compact nanoparticles, and thus

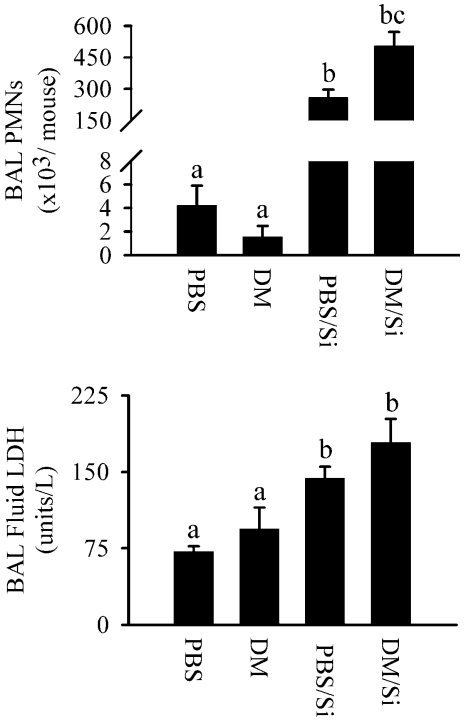

Figure 3. Lung inflammation and damage in response to PBS, DM, PBS/Si or DM/Si, 1 day post-exposure. BAL PMNs (upper panel) were determined as a marker of pulmonary inflammation. BAL fluid LDH (lower panel) were measured as a marker of cytotoxicity. Values are means ± SE ($n = 9$–10). Bars with different letters are significantly different.

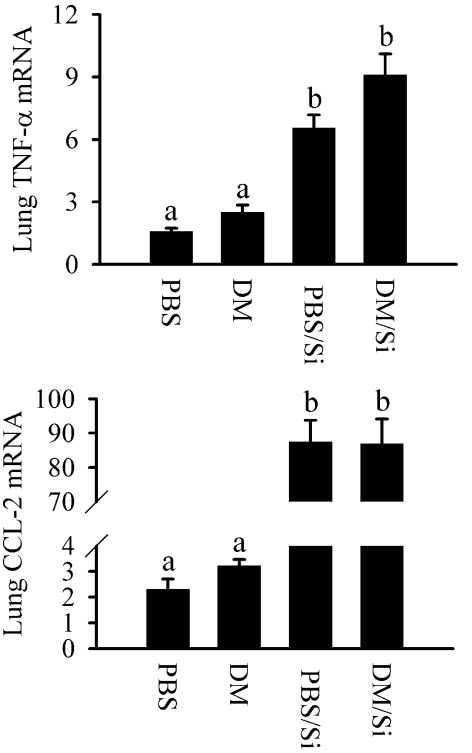

Figure 4. Expression of lung cytokines in response to PBS, DM, PBS/Si or DM/Si, 1 day post-exposure. Lung TNF-α (upper panel) and CCL-2 (lower panel) were determined as markers of inflammatory cytokine expression. Values are means ± SE ($n =$ 5–6). Bars with different letters are significantly different.

represents an alternative nanoparticle dispersion medium to BAL fluid. We also investigated DM as a dispersant for another major class of nanoparticles, carbon nanotubes. Examination and comparison of TEM micrographs of MWCNT suspended in PBS and DM indicate that DM disperses MWCNT substantially better than PBS.

Although these nanoparticles were all better dispersed in DM relative to PBS, they still were not monodisperse. Based on our extensive experience in working with a variety of nanomaterials, we have inferred that conditions necessary to create monodispersed nanoparticles require surfactant concentrations that are physiologically incompatible and beyond the scope of application for *in vivo* studies. Thus, investigators will need to empirically optimize dispersion for their particular studies, but it seems likely that many nanotoxicology studies will use nanoparticles with some degree of agglomeration. A similar conclusion has recently been reached by another laboratory, which investigated various protein-containing solutions for nanoparticle dispersion (Buford et al. 2007).

Having established DM as an effective nanoparticle dispersion vehicle *in vitro*, our objective was to examine the use of DM for *in vivo* toxicology studies. For these *in vivo* studies, we used fine silica as a 'model' particle. The rationale for choosing fine silica and not a nanoparticle to investigate the efficacy of DM as a dispersant is due to the following concern. If a nanoparticle had been used, two independent unknown variables, i.e. particle dispersion and particle coating, would have interfered with the interpretation of the findings. Indeed, studies in our laboratory have determined that one-day after intratracheal instillation, rats exposed to dispersed suspensions of UFTiO$_2$ and UFCB have significantly greater pulmonary inflammation and damage versus non-dispersed suspensions (Shvedova et al. 2007). By using fine silica, where agglomeration is not an issue, we eliminated the particle dispersion variable, allowing us to test whether DM would coat the particles and mask its bioactivity. This is because silica had similar particle size in either PBS or DM, i.e., 0.96 ± 0.06 or 0.97 ± 0.04 μm, respectively, as determined by dynamic light scattering ($n = 3$).

The *in vivo* experiments were conducted to address two questions. The first, and most fundamental, was whether DM causes any pulmonary toxicity when used alone. If DM induced toxicity as a vehicle, it would indicate it was not appropriate to use it for *in vivo* studies. Thus, we compared PBS- and DM-exposed groups for various pulmonary endpoints of toxicity. In the lung, no significant

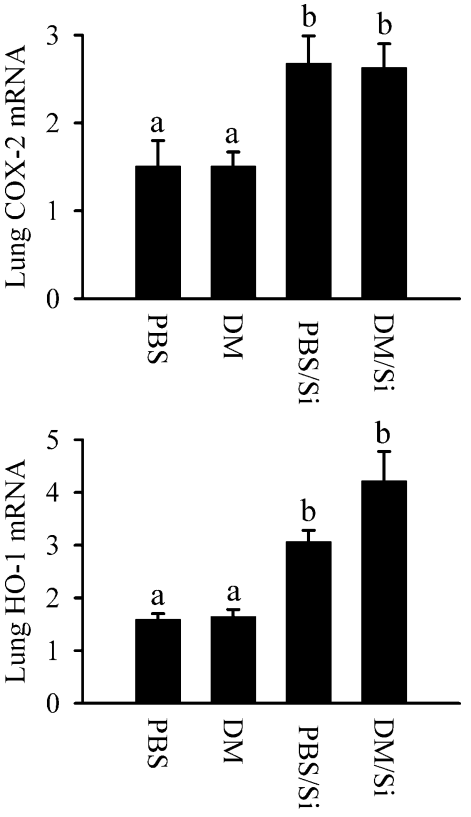

Figure 5. Expression of lung oxidative stress markers in response to PBS, DM, PBS/Si or DM/Si, 1 day post-exposure. Lung COX-2 (upper panel) and HO-1 (lower panel) were determined as markers of oxidative stress. Values are means ± SE ($n = 5$–6). Bars with different letters are significantly different.

changes were observed between either group, as assessed by BAL cell differentials, as well as inflammatory or oxidative stress responses. Taken together, our data indicate that DM does not induce any toxicity in the lung when used as a vehicle.

The second question which was investigated in these *in vivo* experiments was whether DM, by virtue of its containing protein (albumin) and phospholipid (DPPC), would coat the particle and alter its toxicity. Specifically, the concern that DPPC might decrease particle toxicity stems from a previous study which demonstrated that DPPC can essentially render a highly toxic particle like crystalline silica biologically inert, when the DPPC:particle ratio (mg:g) is 1:10 or higher (Keane & Wallace 1995). Thus, we compared the relative toxicities of silica prepared in PBS (PBS/Si) and DM (DM/Si) in mice, to determine if DM would decrease pulmonary toxicity. In the lung, between the BAL studies and measurements of inflammatory and oxidative stress responses, a total of six markers were evaluated. These results did not reveal any overall variations in the toxicological potential of silica prepared in either PBS or DM. The only exception was the observation of a subtle increase in BAL

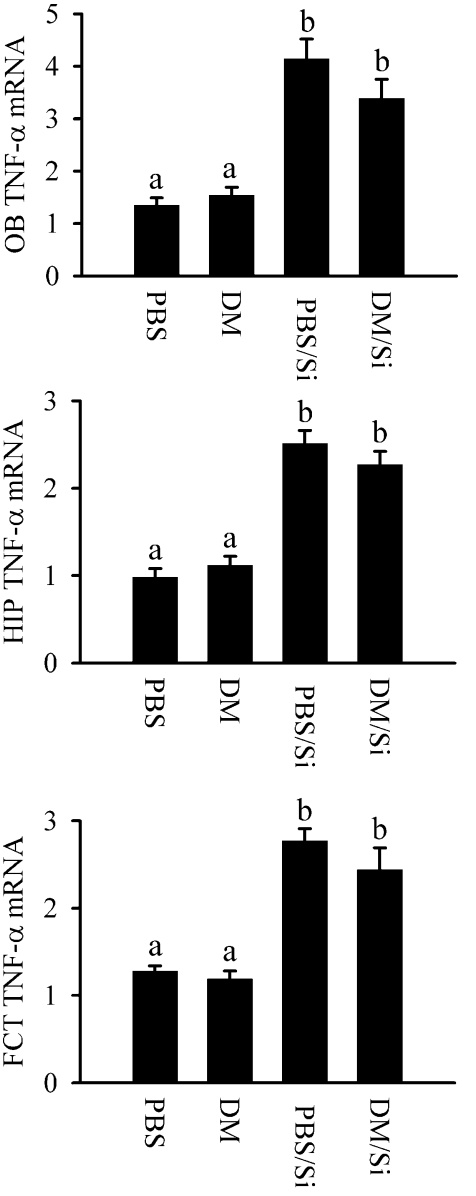

Figure 6. Neuroinflammatory responses in brain one day after pulmonary exposure to PBS, DM, PBS/Si or DM/Si. OB = olfactory bulb (upper panel), HIP = hippocampus (middle panel), and FCT = frontal cortex (lower panel). Values are means ± SE ($n = 8$–13). Bars with different letters are significantly different.

PMNs, but not of other lung indices of toxicity, in DM/Si exposed mice. The physiological or biological relevance of this small effect is not addressed here, and in this study is not consistent with masking the bioactivity of the particle surface.

Overall, the pulmonary data indicate that particle toxicity is not altered by DM. A possible explanation for why DM did not reduce or eliminate silica toxicity is perhaps due the DPPC:particle ratio. The DPPC concentration in our DM is 10 μg/ml and silica was prepared at a concentration of 20 mg/ml of DM, which results in a DPPC:particle

ratio (mg:g) of 1:2000, a value 200 times lower than that previously reported necessary to detoxify silica (Keane & Wallace 1995).

Recent studies have indicated that environmental or occupational exposure to particulate matter and ultrafine particles causes neuroinflammation, cognitive deficits and other central nervous system abnormalities (Hunter & Undem 1999; Oberdörster et al. 2002, 2004; Elder et al. 2006; Shimada et al. 2006; Calderon-Garciduenas et al. 2008). Consequently, our group has initiated research investigating the neurotoxicity of engineered nanomaterials resulting from pulmonary exposure. Comparison of PBS- and DM-exposed groups found no significant neuroinflammatory changes, indicating that DM does not induce any toxicity in the brain when used as a vehicle for pulmonary exposure. In addition, no significant difference in responses to PBS/Si or DM/Si was observed, as assessed by the expression of TNF-α mRNA in selected brain areas, which is consistent with similar measurements in the lung. However, the observation that a significant and consistent increase in TNF-α mRNA occurred in these brain regions in response to either PBS/Si or DM/Si was unexpected, and the mechanism responsible is currently being investigated. Although evidence for accumulation of crystalline silica in the rat brain following inhalation exposure has been reported (Langley et al. 2004), we do not have any evidence to suggest that DM/Si translocation from the lung to the brain is responsible for the neuroinflammation observed in this study.

In conclusion, our *in vitro* studies demonstrate that DM is an effective medium to promote nanoparticle dispersion prior to use for toxicological studies. *In vivo*, when used as a vehicle, DM per se does not elicit toxicity and does not influence or alter toxic responses to silica in either the lung or brain, indicating that it is an effective, biocompatible, and economical vehicle for nanotoxicological evaluation.

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