Pulmonary Instillation Studies with Nanoscale TiO$_2$ Rods and Dots in Rats: Toxicity Is not Dependent upon Particle Size and Surface Area

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Pulmonary toxicology studies in rats demonstrate that nanoparticles administered to the lung are more toxic than larger, fine-sized particles of similar chemistry at identical mass concentrations. The aim of this study was to evaluate the acute lung toxicity in rats of intratracheally instilled pigment-grade TiO$_2$ particles (rutile-type particle size = ~300 nm) versus nanoscale TiO$_2$ rods (anatase = 200 nm x 35 nm) or nanoscale TiO$_2$ dots (anatase = ~10 nm) compared with a positive control particle type, quartz. Groups of rats were instilled with doses of 1 or 5 mg/kg of the various particle types in phosphate-buffered saline (PBS). Subsequently, the lungs of PBS- and particle-exposed rats were assessed using bronchoalveolar lavage fluid biomarkers, cell proliferation methods, and by the histopathological evaluation of lung tissue at 24 h, 1 week, 1 month, and 3 months postinstillation exposure. Exposures to nanoscale TiO$_2$ rods or nanoscale TiO$_2$ dots produced transient inflammatory and cell injury effects at 24 h postexposure (pe) and were not different from the pulmonary effects of larger sized TiO$_2$ particle exposures. In contrast, pulmonary exposures to quartz particles in rats produced a dose-dependent lung inflammatory response characterized by neutrophils and foamy lipid-containing alveolar macrophage accumulation as well as evidence of early lung tissue thickening consistent with the development of pulmonary fibrosis. The results described herein provide the first example of nanoscale particle types which are not more cytotoxic or inflammogenic to the lung compared to larger sized particles of similar composition. Furthermore, these findings run counter to the postulation that surface area is a major factor associated with the pulmonary toxicity of nanoscale particle types.

Key Words: titanium dioxide particles; pulmonary toxicity; nanoscale TiO$_2$ rods and dots; particle size; particle surface area.

Due to their extremely small size, a key area for nanotechnology will be the assessment of health effects and, in particular, lung toxicity to nanomaterials. In this regard, pulmonary toxicity studies in rats demonstrate that ultrafine particles (generally synonymous with the term “nanoparticles” and defined as particles in the size range <100 nm) produce enhanced inflammatory responses when compared to larger sized particles of identical chemical composition at equivalent mass concentrations (Donaldson et al., 2001; Oberdorster et al., 2005). Surface area and particle number determinations appear to play important roles in facilitating ultrafine particle-related lung toxicity. Contributing to the effects of ultrafine particle toxicity is their very high size-specific deposition rate when inhaled experimentally as singlet ultrafine particles rather than as aggregated particles. Some evidence suggests that inhaled ultrafine particles, after deposition in the lung, largely escape alveolar macrophage surveillance and gain greater access to the pulmonary interstitium through translocation from alveolar spaces through the epithelium (Donaldson et al., 2001; Oberdorster et al., 2005).

A limited number of studies in laboratory animals have been reported, which have assessed the inhalation toxicity of ultrafine particles or nanoparticles at very high particle concentrations. Some hazard-based toxicity studies were conducted to investigate the pulmonary effects caused by lung particle overload, i.e., induction of lung tumors in rats at high-retained particulate lung burdens. Specifically, chronic inhalation studies with ultrafine (P25) or fine-sized TiO$_2$ particles (average primary particle sizes ~25 and ~300 nm, respectively) have shown that less than one-tenth the inhaled mass concentrations of the aggregated ultrafine particles, compared with the fine particles, produced equivalent numbers of lung tumors in rats in these 2-year particle overload studies (approximately 16-30%)—primarily benign tumors for fine-sized TiO$_2$ particles (Heinrich et al., 1995; Lee et al., 1985). In addition, shorter term pulmonary toxicity studies with ultrafine particles, as well as fine carbon black, nickel, and TiO$_2$ particles, in rats (Bermudez et al., 2002, 2004; Elder et al., 2005; Ferin et al., 1992; Zhang et al., 2003) have demonstrated an enhanced lung inflammatory potency of the ultrafine particles when compared to fine-sized or larger particulates of similar composition. When the instilled doses were expressed in terms of particle surface area, the responses of the ultrafine and fine TiO$_2$ particles fell on the same dose-response curve (Donaldson et al., 2005).
et al., 2001; Oberdorster et al., 2005). This is because a given mass of ultrafine particles has a much greater surface area when compared to the mass of fine-sized particles and therefore is more likely to cause particle overload in the lung. Thus, from a toxicological and regulatory viewpoint, it will be important to delineate the pulmonary effects of ultrafine particles in rats at overload versus nonoverload conditions.

The results of some mechanistic studies have suggested that the fate of ultrafine particles following deposition in the alveolar regions of the rat lung may differ from that of fine-sized particles. In this regard, the ultrafine particles are not as readily phagocytized by alveolar macrophages as are larger particles and, as a consequence, translocate much more rapidly to interstitial sites.

The aim of this study was to test the hypothesis that nanoscale TiO₂ particulates in the form of nanodots or nanorods produce substantially greater pulmonary inflammation and cytotoxicity when compared to fine-sized titanium dioxide particle types at equivalent mass dose concentrations.

## MATERIALS AND METHODS

### Animals

Groups of male Crl:CD(SD) IGS BR rats (Charles River Laboratories, Inc., Raleigh, NC) were used in this study. The rats were approximately 8 weeks old at the start of the study (mean weights in the range of 240–255 g). All the procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee, and the animal program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

### Particle types

Quartz particles (crystalline silica, Min-U-Sil 5) ranging in size from 1–3 μm (Table 1, Fig. 1) were obtained from Pittsburgh Glass and Sand Corporation (Berkeley Springs, WV). R-100, rutile-type, pigment-grade titanium dioxide particles (i.e., fine sized; ~99 wt% titanium dioxide, ~1 wt% alumina) possessing an average particle size of ~300 nm and an average Brunauer-Emmett-Teller (BET) surface area of ~6 m²/g were obtained from the DuPont Company (Wilmington, DE). Nano-TiO₂ anatase rods were synthesized hydrothermally (in water at 95°C) in low surfactant concentrations (tetramethylammonium hydroxide [TMATH] 0.91 mmol/l). Nano-TiO₂ anatase dots were synthesized hydrothermally (in ethanol at 150°C) with no surfactant present. BET-specific surface areas were determined from N₂ adsorption onto the nanotio₂ powders using a Micrometrics ASAP 2010 apparatus Brumauer et al. (1938). Samples were degassed for several hours prior to the N₂ adsorption analysis, which was carried out at liquid nitrogen temperature (~196°C). Cryo-transmission electron micrographs (cryo-TEMs) were taken on a JEOL 2010 microscope. Each TEM sample was prepared by flash freezing 2 μl of 3 mg/ml nano-TiO₂ solution via FEI Vitrobot at liquid nitrogen temperature (~196°C) onto a 300-mesh copper/carbon grid (Ted Pella, Inc., Redding, CA). Differential thermal analysis was performed in air on a Thermal Advantage SDT 2960 apparatus using a temperature range of 25–600°C and a heating rate of 20°C/min.

### General experimental design

The fundamental features of this pulmonary bioassay are (1) dose-response evaluation and (2) time course assessments to determine the sustainability of any observed effect. Thus, the major endpoints of this study were the following: (1) time course and dose-response intensity of pulmonary inflammation and cytotoxicity; (2) airway and lung parenchymal cell proliferation; and (3) histopathological evaluation of lung tissue.

Groups of rats (5 rats per group per dose per time point) were intratracheally instilled with doses of 1 or 5 mg/kg quartz (crystalline silica) particles, fine-sized TiO₂ particles, TiO₂ nanoscale rods, or TiO₂ nanoscale dots. All particles were prepared in a volume of phosphate-buffered saline (PBS) and subjected to polytron dispersion. Groups of PBS-instilled rats served as controls. The lungs of PBS-exposed and particle-exposed rats were evaluated by bronchoalveolar lavage (BAL) fluid analyses at 24 h, 1 week, 1 month, and 3 months postexposure (pe).

For the morphological studies, additional groups (4 rats per group per high dose per time point; 4 rats per group per low dose for the first two time points) of animals were instilled with the particle types listed above as well as PBS. These studies were dedicated for lung tissue analyses and consisted of cell proliferation assessments and histopathological evaluations of the lower respiratory tract. A preliminary study was conducted with a nearly identical experimental design, i.e., exposures to fine-sized TiO₂ particles or nanoscale TiO₂ dots or rods, with the exception that the crystalline silica group was not included.

For the BAL studies, groups of male rats were exposed via intratracheal instillation to (1) vehicle control—PBS; (2) fine-sized TiO₂ particle types in PBS at 1 or 5 mg/kg; (3) nanoscale TiO₂ rods in PBS at 1 or 5 mg/kg; (4) nanoscale TiO₂ dots in PBS at 1 or 5 mg/kg; or (5) Min-U-Sil crystalline quartz particles in PBS at 1 or 5 mg/kg (second study; see Table 2).

For the morphological studies, additional groups of animals were instilled with the particle types listed above plus the vehicle controls, i.e., PBS. These studies and the corresponding groups of rats were dedicated to lung tissue analyses, but only the high-dose groups (5 mg/kg) and PBS controls were utilized in the morphological studies. These studies consisted of cell proliferation assessments and histopathological evaluations of the lower respiratory tract. Similar to the BAL fluid studies, the intratracheal instillation exposure period was followed by 24-h, 1-week, 1-month, and 3-month recovery periods (Table 2).

### Pulmonary lavage

The lungs of sham and particulate-exposed rats were lavaged with a warmed PBS solution as described previously. Methodologies for cell counts, differentials, and pulmonary biomarkers in lavaged fluids were conducted as previously described (Warheit et al., 1991, 1997). Briefly, the first 12 ml of lavaged fluids recovered from the lungs of PBS or particulate-exposed rats was centrifuged at 700 g, and 2 ml of the supernatant was removed for biochemical studies. All biochemical assays were performed on BAL fluids using a Roche Diagnostics (BMC)/Hitachi 717 clinical chemistry analyzer using the Roche Diagnostics (BMC)/Hitachi reagents. Lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and lavage fluid protein were measured using the Roche Diagnostics (BMC)/Hitachi reagents. LDH is a cytoplasmic enzyme and is used as an indicator of cell injury. ALP activity is a measure of Type II alveolar epithelial cell secretory activity, and increased ALP activity in BAL fluids is considered to be an indicator of Type II lung epithelial cell toxicity. Increases in BAL fluid protein concentrations generally are consistent with enhanced permeability of vascular proteins into the alveolar regions, indicating a breakdown in the integrity of the alveolar-capillary barrier.

### Pulmonary cell proliferation studies

This experiment was designed to measure the effects of particle exposures on airway and lung parenchymal cell turnover in rats following the 24-h, 1-week, and 1- and 3-month pe periods. Groups of particulate-exposed rats and the corresponding controls were pulsed 24 h after installation, as well as 1 week and 1 and 3 months pe, with an intraperitoneal injection of 5-bromo-2’-deoxyuridine (BrdU) dissolved in a 0.5 N

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**TABLE 1**

<table>
<thead>
<tr>
<th>Crystal phase</th>
<th>size [μm]</th>
<th>Surface area [m²/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-100</td>
<td>Rutile</td>
<td>300</td>
</tr>
<tr>
<td>Nanoscale rods</td>
<td>Anatase</td>
<td>92–233</td>
</tr>
<tr>
<td>Nanoscale dots</td>
<td>Anatase</td>
<td>5.8–6.1</td>
</tr>
<tr>
<td>Min-U-Sil quartz</td>
<td>Crystalline</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*aSurface area determined by BET-nitrogen technique.*
Characterization of fine-sized and nano-TiO₂ samples (rods and dots). TEMs of nano-TiO₂ (A) rods, 92–233 nm in diameter, and (B) dots, 5.78–6.10 nm. Samples synthesized with surfactant (rods) are individually separated, with little or no aggregation. However, samples synthesized without surfactant (dots) exhibit moderate to severe aggregation. Surface area and surface charge describe the rods to be 26.54 ± 0.61 m²/g in a neutral solution; for the dots, 169.41 ± 0.64 m²/g in a neutral solution. (C) The XRD patterns for nanocrystalline TiO₂ rods and dots. (D) The Fourier transform infrared spectrum of TMAH surfactant, as compared to the synthesized nano-TiO₂ rods. Spectrum shows that dialysis and filtration methods used in the purification steps of nano-TiO₂ rod production are sufficient to remove surfactant catalyst, to yield a surfactant-free sample. (E) As a control, fine-sized TiO₂ particles (300 nm in diameter) were used. TEM shows morphology and size, as compared to the nano-TiO₂ samples.
sodium bicarbonate buffer solution at a dose of 100 mg/kg body weight. The animals were euthanized 6 h later by pentobarbital injection. Following cessation of spontaneous respiration, the lungs were infused with a neutral buffered formalin fixative at a pressure of 21 cm H2O. After 20 min of fixation, the trachea was clamped, and the heart and lungs were carefully removed en bloc and immersion fixed in formalin. In addition, a 1-cm piece of duodenum (which served as a positive control) was removed and stored in formaldehyde. Subsequently, parasagittal sections from the right cranial and caudal lobes and regions of the left lung lobes as well as the duodenal sections were dehydrated in 70% ethanol and sectioned for histology. The sections were embedded in paraffin and cut and mounted on glass slides. The slides were stained with an anti-BrdU antibody, with a 3-amino-9-ethyl carbazole marker, and counterstained with aqueous hematoxylin. A minimum of 1000 cells per animal were counted each in the terminal bronchiolar and alveolar regions. For each treatment group, immunostained nuclei in airways (i.e., terminal bronchiolar epithelial cells) or lung parenchyma (i.e., epithelia, interstitial cells, or macrophages) were counted by light microscopy at a magnification of ×1000 (Warheit et al., 1991, 1997).

Morphological/histopathology studies. The lungs of rats exposed to particulates or PBS controls were weighed and prepared for microscopy by airway infusion under pressure (21 cm H2O) at 24 h, 1 week, and 1 and 3 months pe. Sagittal sections of the left and right lungs were made with a razor blade. Tissue blocks were dissected from left, right upper, and right lower regions of the lung and were subsequently prepared for light microscopy (paraffin embedded, sectioned, and hematoxylin-eosin stained; Warheit et al., 1991, 1997).

Statistical analyses. For analyses, each of the experimental values were compared to their corresponding sham control values for each time point. A one-way ANOVA and Bartlett’s test were calculated for each sampling time. When the F-test from ANOVA was significant, the Dunnett test was used to compare means from the control group and each of the groups exposed to particulates. Significance was judged at the 0.05 probability level.

RESULTS

BAL Fluid Results

Pulmonary inflammation. The numbers of cells recovered by BAL from the lungs of high-dose quartz-exposed (5 mg/kg) groups were substantially higher than any of the other groups for all pe time periods (Fig. 2). Intratracheal instillation exposures to fine- or nano-sized TiO2 particle types (dots or rods) produced a transient, short-lived, pulmonary inflammatory response, as evidenced by an increase in the percentages/numbers of BAL-recovered neutrophils, measured 24 h pe but not sustained at other times pe. In contrast, intratracheal

![FIG. 2. Numbers of cells recovered in BAL fluids from particulate-exposed rats and the corresponding controls at 24 h, 1 week, and 1 and 3 months pe. Values given are means ± SDs. The numbers of cells recovered by BAL from the lungs of high-dose quartz-exposed (5 mg/kg) groups were higher than any of the other groups for all pe time periods.](image-url)
instillation exposures to quartz particles (1 and 5 mg/kg) produced sustained pulmonary inflammatory responses, as measured through 3 months pe (Fig. 3). Cytocentrifuge preparations of lavaged cells recovered from rats exposed to TiO2 nanoscale dots at 24 h (Fig. 4A) and 1 week (Fig. 4B) pe demonstrate the transient inflammatory response at 24 h and recovery at 1 week, as well as the wide distribution of “aggregated” nano-dots phagocytized by alveolar macrophages.

**BAL fluid parameters.** Transient and reversible increases in BAL fluid LDH values were measured in the lungs of rats exposed to the high dose (5 mg/kg) of TiO2 nanoscale rods at 24 h pe but were not sustained through the other pe time periods. In contrast, exposures to 5 mg/kg quartz particles produced a persistent increase in BAL fluid LDH values throughout the 3-month pe period (Fig. 5). Transient but not significant increases in BAL fluid microprotein values were measured in the lungs of high-dose (5 mg/kg) nanoscale TiO2 rod–exposed rats at 24 h pe and were not significantly different from controls at any pe time period. In contrast, exposures to 5 mg/kg quartz particles produced sustained increases in BAL fluid microprotein values at 24 h, 1 week, and 1 and 3 months pe (Fig. 6). No significant increases in BAL fluid ALP values were measured among any of the groups at any time period pe (data not shown).

To summarize the results from the BAL fluid biomarker studies, pulmonary exposures to quartz particles (1 and 5 mg/kg) produced sustained pulmonary inflammatory responses, as measured through 3 months pe; *p < 0.05.

**Lung Tissue Studies**

**Lung weights.** Lung weights of rats were increased with increasing age on the study (i.e., increased pe time periods following instillation). Lung weights in high-dose quartz-exposed rats were slightly increased versus controls at 1 week and 1 month pe and substantially increased at 3 months pe (data not shown).

**Cell proliferation results.** Tracheobronchial cell proliferation rates (percent immunostained cells taking up BrdU) were measured in high-dose (5 mg/kg), particulate-exposed rats and the corresponding controls at 24 h, 1 week, and 1 and 3 months pe. Although a nonsignificant increase in cell-labeling indices was noted in low-dose fine TiO2–exposed lungs at 1 week, this effect was not measured in rats exposed to higher doses at any time period. Transient increases in cell-labeling indices were measured in high-dose quartz-exposed animals at 24 h pe, but these effects were not sustained (data not shown).

Lung parenchymal cell proliferation rates (percent immunostained cells taking up BrdU) were measured in high-dose (5 mg/kg), particulate-exposed rats and the corresponding
controls at 24 h, 1 week, and 1 and 3 months pe. Small but transient increases in lung cell proliferation indices were measured in the low- and high-dose fine TiO₂-exposed rats at 1 week pe, but these effects were not sustained. In addition, this increase did not correlate with BAL fluid or histopathological results at the 1-week pe time point and has not been measured in previous studies under similar experimental conditions. Significantly larger increases in cell proliferation indices were measured in the lungs of quartz-exposed rats at 1 and 3 months pe (Fig. 7).

To summarize the results of airway and alveolar cell proliferation studies, when compared to other particle types or controls, pulmonary exposures to 5 mg/kg quartz particles produced higher lung parenchymal cell proliferation rates at 1 and 3 months pe, suggesting a greater likelihood to result in adverse pulmonary effects over time with continued exposures.

**Histopathological evaluation.** Histopathological analyses of lung tissues revealed that pulmonary exposures to fine-sized TiO₂ particles, TiO₂ nanoscale rods, or to TiO₂ nanoscale dots produced no significant adverse effects when compared to the PBS-exposed controls, as evidenced by the normal lung architecture observed in the exposed animals at postinstillation exposure time periods ranging from 24 h to 3 months. Accumulations of TiO₂-containing macrophage aggregates were observed in the lung tissue of rats exposed to fine-sized or nanoscale TiO₂ rods or dots.

Histopathological analyses of lung tissues revealed that pulmonary exposures to quartz particles in rats produced dose-dependent lung inflammatory responses characterized by neutrophils and foamy (lipid containing) alveolar macrophage accumulation. In addition, lung tissue thickening as a prelude to the development of fibrosis was evident and progressive (Fig. 8).

**DISCUSSION**

The objective of this pulmonary bioassay study was to assess the acute lung toxicity of intratracheally instilled, nanoscale TiO₂ rods or dots in rats. Using pulmonary bioassay methodology, the lung toxicity of instilled nanoscale TiO₂ rods or dots were compared with a positive control particle type, quartz, as well as a negative control particle type, fine TiO₂ particles. The specific aim was to determine whether exposures to nanoscale titanium dioxide particle types could produce enhanced pulmonary inflammation in rats when compared to fine-sized TiO₂ particles, a particle type known to have low toxicity. Pulmonary instillation exposures to TiO₂ nanoscale rods or nanodots produced no significant adverse pulmonary effects. Results from the BAL fluid and cell proliferation evaluations demonstrated that pulmonary exposures to quartz particles, particularly at higher dose, produced significant adverse effects versus controls in pulmonary inflammation, cytotoxicity, and lung parenchymal cell proliferation indices. In contrast, nanoscale TiO₂ rods or dots produced only transient and reversible inflammation, due primarily to the method of exposure (intratracheal instillation) rather than to the effects of the nanoscale TiO₂ particulates per se in the lung. Similarly, exposures to fine TiO₂ particles or the vehicle, PBS, resulted only in short-term and reversible lung inflammation, also likely related to the effects of the instillation procedure. Histopathological evaluation demonstrated that quartz particle exposures produced pulmonary inflammation, foamy macrophage accumulation, and tissue thickening (i.e., fibrosis). Exposures to nanoscale TiO₂ rods or dots produced minor adverse tissue reactions in the lungs, including macrophage accumulations, normal responses to high dust concentrations.
Numerous studies in rats and the corresponding reviews on the topic have concluded that exposures to nanoscale or ultrafine particles (generally defined as particles in the size range \(<100\) nm) produce greater inflammatory and cytotoxic effects when compared to exposures to larger sized particles at equivalent mass concentrations (Donaldson et al., 2001; Oberdorster et al., 2005). Surface area metrics appear to play an important role in the development of nanoscale or ultrafine toxicity. Some evidence suggests that two main mechanisms may be operative in the development of nanotoxicity which delineates these particle types from their bulk counterparts. These effects are postulated to be (1) the greater potential for nanoparticles to escape macrophage surveillance and, as a consequence, translocate from airspace to interstitium or vascular sites and thus cause either interstitial inflammation/fibrosis or translocate to other organs; (2) the direct effect of nanoparticles on cells to cause injury and oxidative stress (Donaldson et al., 2001; Oberdorster et al., 2005). Surface area metrics appear to play an important role in the development of nanoscale or ultrafine toxicity. Some evidence suggests that two main mechanisms may be operative in the development of nanotoxicity which delineates these particle types from their bulk counterparts. These effects are postulated to be (1) the greater potential for nanoparticles to escape macrophage surveillance and, as a consequence, translocate from airspace to interstitium or vascular sites and thus cause either interstitial inflammation/fibrosis or translocate to other organs; (2) the direct effect of nanoparticles on cells to cause injury and oxidative stress (Donaldson et al., 2001; Oberdorster et al., 2005).

In the current studies described herein, the surface areas of the three titanium dioxide particle types were the following: \(6\) m\(^2\)/g for the fine TiO\(_2\) samples; \(~26\) m\(^2\)/g for the TiO\(_2\) nanorods; and \(169\) m\(^2\)/g for the TiO\(_2\) nanodots—a 4-fold and nearly 30-fold difference in surface area properties. Moreover, no differences were measured in the pulmonary responses to anatase nanodots versus anatase nanorods—despite a sixfold difference in surface area properties. It should be noted that the crystal phases of the TiO\(_2\) particle types tested, i.e., fine TiO\(_2\) (rutile) and TiO\(_2\) nanorods and TiO\(_2\) nanodots (anatase), were different. Moreover, the interspecies studies on lung responses to TiO\(_2\), reported by Bermudez et al. utilized rutile-type pigment-grade TiO\(_2\) particles (Bermudez et al., 2002) as well as “ultrafine” TiO\(_2\) particles (Bermudez et al., 2004) which are comprised of 80% anatase and 20% rutile. Thus, the comparisons of pigment-grade and ultrafine TiO\(_2\) particle toxicity do not represent comparisons of TiO\(_2\) particles of identical composition (i.e., there was an additional variable in this comparison [crystal phase], other than particle size).

With regard to the different crystal structures of TiO\(_2\) particles, Sayes et al. (submitted manuscript) recently assessed the photocatalytic properties, reactive oxygen species generation, and \textit{in vitro} cytotoxic potentials of nano-TiO\(_2\) particles of three different crystal phases, namely, anatase, anatase/rutile, and rutile.

**FIG. 5.** BAL fluid LDH values for particulate-exposed rats and the corresponding controls at 24 h, 1 week, 1 month, and 3 months pe. Values given are means ± SDs. Transient and reversible increases in BAL fluid LDH values were measured in the lungs of rats exposed to high-dose (5 mg/kg) TiO\(_2\) nanoscale rods at 24 h pe but were not sustained through the other pe time periods. In contrast, exposures to 5 mg/kg quartz particles produced a sustained increase in BAL fluid LDH values through the 3-month pe period; \(*p < 0.05.\)
and rutile phases. The particle sizes of the three nanoparticle types were similar, ranging from 3 to 10 nm, with surface areas ranging from 112 to 153 m²/g. These investigators reported that, when compared to the other crystal phases, the nano-TiO₂ particles in the anatase phase, produced the most reactive oxygen species generation and the largest cytotoxic responses following *in vitro* exposures to human dermal fibroblasts or to A549 human lung epithelial cells. Sayes *et al.* concluded that

![Graph of BAL fluid MTP values for rats exposed to fine and nano-sized TiO₂ particulates](image1.png)

**FIG. 6.** BAL fluid protein (microprotein assay [MTP]) values for particulate-exposed rats and the corresponding controls at 24 h, 1 week, 1 month, and 3 months post-exposure (pe). Values given are means ± SDs. No significant increases versus controls were measured in any of the nano- or fine-sized TiO₂ particulate groups at any time point post-exposure. In contrast, exposures to 5 mg/kg quartz particles produced sustained increases in BAL fluid microprotein values at 24 h, 1 week, 1 and 3 months post-exposure; *p < 0.05.

![Graph of Lung Parenchymal Cell Proliferation rates of rats exposed to TiO₂ Nano-dots/rods and other particulates](image2.png)

**FIG. 7.** Lung parenchymal cell proliferation rates (BrdU) in particulate-exposed rats and the corresponding controls at 24 h, 1 week, 1 month, and 3 months post-exposure (pe). Values given are means ± SDs. Small but significant transient increases in lung cell proliferation indices were measured in the fine-sized TiO₂-exposed rats at 1 week. Significantly larger increases in cell proliferation indices were measured in the lungs of quartz-exposed rats through 3 months post-exposure; *p < 0.05.
the nano-TiO$_2$ particles in the anatase crystal phase are a superior photocatalyst to the rutile particle types because of differences inherent in the crystal structures of the two phases and not because of differences in surface area. It remains to be determined whether similar results will be measured under in vivo conditions.

With regard to the issue of particle aggregation, it should be noted that even though the TEM in Figure 1B shows extreme aggregation among the nanoscale TiO$_2$ dots, the BET (surface area methodology) and x-ray diffraction (XRD) data demonstrate that these dots maintain a very large surface area (i.e., $>$100 m$^2$/g). Surface area is the nanoproperty that is one of the two main variables of investigation in this study (the other is crystal structure—anatase vs. rutile). As the diameter of the nanoparticle decreases, the surface area increases exponentially. Therefore, precise and accurate tests need to be performed on the surface area of these particle samples. This was conducted using two independent methods—BET and XRD. BET uses a flow of argon or nitrogen gas to suspend, then absorb to, each individual particle. XRD is used to determine the crystalline phase, and also by examining the line widths of each peak and employing calculations, both diameter and surface area can be measured.

Primary particle size considerations may sometimes be misleading, particularly when one considers the impacts of aggregation of small particles. In this regard, it is interesting to note that Bermudez conducted two 90-day interspecies comparison studies (rats, mice, and hamsters) (1) with aerosols of pigment-grade TiO$_2$ particles and (2) with aerosols of ultrafine P25 TiO$_2$ particles. The pigment-grade TiO$_2$ particles have a primary particle size of ~300 nm (Bermudez et al., 2002), while the P25 TiO$_2$ particles have an average primary particle size of 21 nm (Bermudez et al., 2004). The mass median aerodynamic diameter (MMAD), i.e., particle size of the pigment-grade aerosol in the exposure chamber, ranged from 1.36–1.44 $\mu$m, while the MMAD for the ultrafine aerosol was calculated to be 1.37 $\mu$m, suggesting aggregation of both fine and P25 ultrafine TiO$_2$ particles. Yet, the potency of the P25 to cause inflammation and cytotoxicity in the exposed rats was 5–10 times greater (on a mass basis) when compared to the effects of the pigment-grade TiO$_2$ particles. Since the P25 ultrafine TiO$_2$ particles have a measured surface area of ~55 m$^2$/g (BET), while the pigment-grade TiO$_2$ particles have a surface area of ~6 m$^2$/g, these results are consistent with the notion that when the inhaled doses are expressed in terms of surface area, the responses of the ultrafine and fine TiO$_2$ particles fall on the same dose-response curve. Nonetheless, it is intriguing to wonder whether the aggregated ultrafine P25 TiO$_2$ particles became “disaggregated” following inhalation and the corresponding deposition in the alveolar regions of the lung, and this might account for their enhanced inflammatory potency. As a consequence, it can be postulated that (1) the ultrafine TiO$_2$ particles became disaggregated following inhaled particle deposition in the lung and/or (2) the ultrafine TiO$_2$ particles may have remained in an aggregated state but still had a maximum effect in producing lung inflammation and cytotoxic effects. Thus, it remains to be determined whether particle aggregation plays a significant role in mitigating the presumed effects of particle size—particularly since the particle surface area does not appear to change following aggregation.

The degree to which engineered nanoparticles aggregate in the ambient aerosol or occupational environment and subsequently do or do not disaggregate following inhalation and particle deposition in the lung will strongly influence particle deposition rates and patterns as well as interactions with lung cells. If the ultrafine/nanoparticles disaggregate upon interaction with alveolar lung fluids at the sites of particle deposition (i.e., alveolar duct bifurcations), then they could behave as discrete individual nanoparticles and may stimulate enhanced inflammatory cell recruitment and/or the particles could preferentially translocate to more vulnerable anatomical compartments of the lung (e.g., interstitium). Alternatively, aggregated nanoparticle types could behave as fine-sized particles.

In conclusion, using a pulmonary bioassay, the acute lung toxicity of intratracheally instilled hydrophilic, nanoscale TiO$_2$ particulate types were compared with a positive control particle type, quartz, as well as a negative reference particle type, fine-sized TiO$_2$ particles. In addition, the results of these instillation studies were compared with quartz particles as a positive reference material. The results presented herein demonstrate that intratracheally instilled, 5 mg/kg doses of nanoscale TiO$_2$ particle types did not produce significant lung toxicity in rats—results similar to those derived from fine-sized titanium dioxide samples. At similar doses, exposures to quartz particles produces a sustained pulmonary inflammatory response in rats,
leading to the development of pulmonary fibrosis and other adverse lung effects. In general, pulmonary toxicology studies have demonstrated that nanoparticles produce increased pulmonary inflammation and cytotoxicity when compared to bulk particle types of similar composition. The results presented in our study with TiO\textsubscript{2} nanoscale rods and nanoscale dots are at variance with this “conventional wisdom.” In this regard, it is unclear why the results presented in this study differ from earlier findings of other investigators. However, it is noteworthy that two different crystal structures of TiO\textsubscript{2} particles were evaluated in this study (anatase and rutile), indicating that more than one variable (i.e., particle size and surface area) was evaluated. Moreover, a paucity of toxicology studies have been conducted which have systematically examined the role of particle size and surface area in producing lung toxicity. This ultrafine/nanoparticle toxicity database needs to be expanded before any general conclusions regarding particle size can be promoted.

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