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BIOLOGICAL RESPONSE TO NANO-SCALE TiO₂: ROLE OF PARTICLE DOSE, SHAPE AND RETENTION

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Abstract

TiO₂ is one of the most widely used nanomaterials, valued for its highly refractive, photocatalytic and pigmenting properties. TiO₂ is also classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen. The objectives of this study were to establish a lowest observed effect level (LOEL) for nano-scale TiO₂, determine TiO₂ uptake in the lungs, and estimate toxicity based on physico-chemical properties and retention in the lungs. *In vivo* lung toxicity of nano-scale TiO₂ using varying forms of well-characterized, highly-dispersed TiO₂ was assessed. Anatase/rutile P25 spheres (TiO₂-P25), pure anatase spheres (TiO₂-A), and anatase nanobelts (TiO₂-NB) were tested. To determine the effects of dose and particle characteristics, male Sprague-Dawley rats were given TiO₂ (0, 20, 70, or 200 µg) via intratracheal instillation. Broncho-alveolar lavage fluid (BALF) and lung tissue were obtained for analysis 1 and 7 days post exposure. Despite abundant TiO₂ inclusions in all exposed animals, only TiO₂-NB elicited any significant degree of inflammation seen in BALF at the 1-day time-point. This inflammation resolved by 7 days; although, TiO₂ particles had not cleared from alveolar macrophages recovered from the lung. Histological examination showed TiO₂-NB caused cellular changes at day 1 which were still evident at day 7. We conclude TiO₂-NB is the most inflammatory with a lowest observable effect level of 200 µg at 1 day post instillation.

Keywords

Titanium dioxide particles; pulmonary toxicity; inflammation; lung dosimetry; engineered nanomaterial

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INTRODUCTION

Titanium dioxide (TiO₂) is one of the most widely used nano-scale materials to date, yet it is also classified by the International Agency for Research on Cancer (IARC) as a possible carcinogen to humans. Valued for its highly-refractive, photocatalytic and pigmenting properties, the conversion of bulk to nano-scale TiO₂ for consumer products (e.g. topical sunscreens and cosmetics) and industrial products (e.g. paints and coatings) is rapidly increasing (Robichaud et al. 2009). Thus, there is high probability for TiO₂ exposure, especially in the workplace based on mass production and inhalation exposure scenarios (Kuhlbusch et al. 2011).

Investigators report conflicting results regarding TiO₂ toxicity. Nano-scale TiO₂ has been observed to cause pulmonary inflammation in rats and mice *in vivo* (Bonner et al. 2013; Hougaard et al. 2010; Ma-Hock et al. 2009; McKinney et al. 2012; Warheit et al. 2006), and a number of *in vitro* studies suggest that TiO₂ engineered nanomaterials can elicit oxidative stress and DNA damage (Cui et al. 2012; Meena et al. 2012). It has been postulated nano-TiO₂ can cross the placental barrier resulting in reduced daily sperm production (Takeda et al. 2009) and altered neurological development (Hougaard et al. 2010; Shimizu et al. 2009; Takeda et al. 2009) in exposed offspring. However, there are a number of studies demonstrating no adverse pathology associated with TiO₂ exposure. For example, Horie et al. did not find lung inflammation or oxidative stress in rats exposed intratracheally to fine or nano-scale TiO₂ (2012), and Saber et al. did not find DNA damage after instillation of nano-scale TiO₂ (2012).

The lack of congruent findings across nano-scale TiO₂ studies is challenging for regulators charged with the task of protecting health and welfare because promulgation of exposure standards requires predictable dose-response patterns proven reliable by multiple studies. The objectives of the present study are to establish a lowest observed effect level (LOEL) for nano-scale TiO₂ and better understand TiO₂ nano-scale toxicity based on physico-chemical characteristics and retention. Three different forms of well characterized, highly dispersed TiO₂ nanoparticles, anatase/rutile P25 spheres (TiO₂-P25), pure anatase spheres (TiO₂-A), and anatase nanobelts (TiO₂-NB), were intratracheally instilled into the lungs of adult male Sprague Dawley rats. Acute lung toxicity was evaluated 1 and 7 days post exposure. LOEL was determined with doses ranging from 0–200 µg since several *in vivo* studies show intratracheal instillation of nano-scale TiO₂ at a dose of ~1 mg/kg of bodyweight causes acute pulmonary inflammation (Nemmar, Melghit, and Ali 2008; Rehn et al. 2003; Renwick et al. 2004; Sager, Kommineni, and Castranova 2008; Warheit, Webb, Reed, et al. 2007). The dose ranged from 0–0.5 mg/kg of bodyweight per adult male Sprague Dawley rat (~0.4 kg). Inflammation and particle retention with time post-exposure were compared among the three types of nanoparticles. We hypothesized that the physico-chemical particle characteristics of TiO₂ (e.g. shape, phase and structure) modulate the dose-dependent inflammation and cellular injury observed in the respiratory tract.

METHODS

Physicochemical Characterization of TiO₂ Nanoparticles

A full description of the physico-chemical characterization of the three forms of TiO₂ nanoparticles has been recently described by Xia and colleagues (2012). Three distinct TiO₂ forms, anatase/rutile P25 spheres (TiO₂-P25), pure anatase spheres (TiO₂-A), and anatase nanobelts (TiO₂-NB), were tested. TiO₂-P25 (81% anatase and 19% rutile) were obtained from Evonik (Parsippany, NJ). Flame Aerosol Reactor (FLAR)-synthesized TiO₂-A were provided by Dr. Pratim Biswas (Department of Energy, Environmental & Chemical Engineering, Washington University, St. Louis, MO). TiO₂-NB were attained from the laboratory of Dr. Nianqiang Wu (Mechanical and Aerospace Engineering, West Virginia University, Morgantown, WV), as described previously (Hamilton et al. 2009). Scanning electron microscope (SEM) images of all three particle types are illustrated in Figure 1. Surface area measured by BET (Brunauer, Emmett, & Teller) was 53, 173, and 18 m²/g for TiO₂-P25, TiO₂-A, and TiO₂-NB, respectively. The specific TiO₂ forms were chosen for analysis because P25 has been used as the *de facto* standard titania photocatalyst and reported in more than one thousand papers since 1990 (Ohtani 2010). Furthermore, TiO₂ toxicity is thought to increase with anatase content (Warheit, Webb, Reed, et al. 2007) and aspect ratio (Hamilton et al. 2009).

Animal Protocol

Outbred male Sprague-Dawley rats (Harlan Laboratories, Inc., Hayward, CA), 9–10 weeks of age, were used for all experiments. Sprague-Dawley rats were selected based on their extensive use in experimental studies, ease in handling, and high tolerance to intratracheal instillation procedures. Upon arrival, animals were weighed and housed in pairs, in plastic cages (~20" × 16" × 8 ¼") with narrow, straight-wire, stainless steel lids, enrichment objects, and CareFRESH® absorptive paper bedding. Animals were acclimated for one week and given access *ad libitum* to a standard commercial, block-type food and water throughout the duration of the experiment. Animal bedding was replaced and cages were changed on a weekly basis. Aseptic animal rooms were maintained at 22°C with 12-hour dark/light cycles.

Animal weights were recorded throughout the study. Prior to exposure, animals were assigned to treatment groups by weight. This was done in a manner ensuring the mean weight for all treatment groups for day and particle type was statistically the same. Sentinel rats were maintained in the same room and tested to ensure experimental animals remained free of pathogens and/or parasites. UC Davis Institutional Animal Care and Use Committee (IACUC) approval was obtained for all procedures involving rats in this study.

Preparation of TiO₂ Suspensions

Dry TiO₂ nanoparticle stock samples were stored in a desiccator under aseptic conditions at room temperature until needed. All solid-phase TiO₂ used for the nanoparticle suspensions were measured using a calibrated, analytical microbalance with a minimum limit of detection equal to 0.003 mg (M5P Filter Microbalance, Sartorius, Goettingen, Germany). TiO₂ engineered nanomaterials were suspended in dispersion media (DM) prepared as

previously described (Porter et al. 2008). This media was chosen for its similarity to the endogenous fluid of the lung, and its ability to effectively disperse and prevent agglomeration of similar TiO₂ particles without affecting particle toxicity. To summarize, distearoylphosphatidylcholine (DSPC: Sigma-Aldrich), low-endotoxin rat serum albumin (Sigma-Aldrich), 0.9% sterile saline, and 100% ethanol were used to make the DM. Though dipalmitoylphosphatidylcholine (DPPC) was used by Porter, and distearoylphosphatidylcholine (DSPC) was used herein, the two are similar membrane lipids that contain two saturated symmetric hydrocarbon chains. The main difference is that DPPC has a C16 carbon tail, and DSPC has a C18 carbon tail. Because it is the choline group present on both molecules that is charged and acts as the surfactant, the two were essentially interchangeable for our purposes. Proportions were such that in 1 mL of DM there were 0.399 mL of sterile saline, 0.600 mL of rat serum albumin (1 mg RSA powder:1 mL 0.9% saline), and 0.001 mL of DSPC (10 mg DSPC powder: 1 mL ethanol). Suspensions of the highest dose (200µg/250µL) were created by adding DM to a Falcon™ tube with a known mass of TiO₂-P25, TiO₂-A, or TiO₂-NB. Suspensions of spherical TiO₂ were probe-sonicated (Qsonic, LLC, Newton, CT) for 30 minutes using a 10-second on/off duty cycle to ensure adequate particle dispersal. TiO₂ nanobelt suspensions were continuously stirred for 30 minutes with a magnetic micro stir bar to prevent breakage of the delicate structures. Direct dilutions of the 200 µg/250µL suspensions were done with DM to make lower dose suspensions of TiO₂ spheres (10 and 70 µg/250µL) as well. These dilutions were also sonicated using the aforementioned protocols. TiO₂ nanobelts were tested only at the highest dose due to constraints on animal supply and time. The DM alone served as the sham control instillate. To assess potential passivation of particles by the dispersion media, high-dose (200 µg/250µL) suspensions of TiO₂ in phosphate buffered saline (PBS), and sterile 0.9% saline were made for comparisons to PBS, and saline sham controls, respectively, and to high-dose TiO₂ suspensions in DM. All suspensions were prepared and loaded into 1cc Monoject® syringes fitted with 1.5 inch, 22 gauge, blunt-tipped Monoject® needles directly before instillation. Sonication, syringe loading, and instillation were timed to ensure the highest degree of particle dispersion.

Intratracheal Instillation

Food was removed from the animal cages two hours prior to instillation. Each animal was placed in a plexiglass anesthesia chamber and exposed to gaseous Isoflurane for 2 minutes at a rate of 1 liter/minute. Once an animal was sufficiently sedated, it was removed from the chamber and instilled with 0, 10, 70 or 200 µg of TiO₂ using an intratracheal instillation board. Care was taken to deliver the instillate upon the inhalation breath to enhance uniform particle delivery to the lungs. Animals were returned to their cages and allowed to recover with free access to food and water. During recovery, animals were monitored for any signs of distress. For each TiO₂ type, a sample size of 48 rats was used, with six animals per dose (including controls) and 24 rats per time-point (1 and 7 days post-exposure).

Collection and Analysis of BALF and Lung Tissue Samples

Animals were weighed and euthanized with an intraperitoneal injection of Beuthanasia-D (65 mg/kg) at 1 or 7 days post TiO₂ exposure. Broncho-alveolar lavage (BAL) was then performed. Briefly, the trachea was cannulated with a 16-gauge cannula. Five separate 5 mL

aliquots of 0.9% sterile saline were slowly instilled into the lungs using a single 6 mL Monoject® syringe. Aliquots one and two were combined in a single 15 mL Falcon™ tube; while, aliquots three to five were combined in a 50 mL Falcon™ tube. Tubes were centrifuged at 4°C and 2000 rpm for 10 minutes (Beckman Coulter, Inc.). Supernatant from the 15 mL Falcon™ tube was collected for same-day protein and LDH analyses, which were performed using kits from Thermo Scientific (Rockford, IL) and Sigma-Aldrich (St. Louis, MO), respectively. The supernatant from the 50 mL Falcon™ tube was discarded. Cells from the 15 mL Falcon™ tube were resuspended in 10 mL 0.9% sterile saline and combined with those of the 50 mL Falcon™ tube for determination of total cell numbers and cell viability with a hemocytometer and Trypan Blue exclusion dye, respectively. Cell differentials and particle uptake/retention (i.e. visible TiO₂ inclusions in macrophages) were determined by Brightfield microscopy by counting a minimum of 500 cells from a 100 mL cytopsin slide stained with Diff Qwik® (Dade Behring Inc., Newark, DE). Blind analysis was completed by one individual and then compared to results obtained by a second person with similar training to ensure minimal observer bias.

In addition to BALF, histological samples were collected from each animal. After the whole lung was lavaged for BALF collection, the right lobes were tied off with surgical thread and removed. Each right lobe was frozen at -80°C in a separate NUNC™ cryovial for inductively-coupled plasma mass spectrometry (ICP-MS) analysis and/or other future assays. The left lung was fixed hydrostatically (30 cm) using 4% paraformaldehyde (PF) for one hour then stored in the same fixative. After 48 hours, tissue stored in buffered formalin was micro-dissected such that two complimentary halves representing the mediastinal and intercostal portions of the lung were prepared. Airway generations along the main axial pathway were mapped, and approximately six transverse slices (proximal to distal) were made starting from the lobar bronchus. Tissue slices were then dehydrated in a series of graded ethanol, embedded in paraffin, and sectioned to 5 µm with a microtome (HM 355, Microm, Walldorf, Germany). Lung sections were placed on slides, stained with hematoxylin (Harris Hematoxylin, American MasterTech, Lodi, CA) and eosin (Eosin Y Stain, American MasterTech, Lodi, CA), and coverslipped for observations using Brightfield microscopy. Histopathological evaluations were made of cellular changes and inflammation for all regions of the lungs with special emphasis on the examination of terminal bronchioles, bronchoalveolar duct junctions, and alveoli.

Total cell counts and cell differentials along with lactate dehydrogenase (LDH) and Bradford (protein) assays were performed on the BALF as they are common, relatively simple, and inexpensive tests for lung cytotoxicity. Brightfield microscopy of BAL cells and ICP-MS of tissue samples were used to determine TiO₂ retention in the lungs.

ICP Analysis

To pinpoint the region of the lungs with the greatest deposition/retention of particles, ICP-MS analysis was performed on right cranial and caudal lung lobes from the sham control and 200 µg TiO₂ instillation exposures. Particle loading was compared between the cranial lobe and caudal lobe because they represent short and long airway paths of the lung, respectively. ICP-MS allowed for highly sensitive and precise detection of TiO₂; the limit of

detection was 600 ng of titanium. ICP-MS procedures were similar to those previously applied for analysis of titanium content in food, personal care products and paints (Weir et al. 2012). Complete details for digesting titanium dioxide in lung tissues is described elsewhere (Weir 2011). Briefly, tissues were weighed and dried at 80°C until weight loss ceased (~12–18 hours). Subsequently, a microwave digestion method using high purity nitric and hydrofluoric acids was implemented to digest lung tissues and other organic matrices. Next, the liquid portion of each sample was evaporated using a hotplate (160°C, 3–4 hours), leaving only TiO₂, which was re-suspended in a dilute nitric acid solution for ICP-MS analysis. ICP-MS results were subsequently confirmed by ICP-optical emission spectrometry (OES).

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). JMP statistical software (Cary, NC) was used to perform analysis of variance (ANOVA) and post hoc Tukey's tests, or Wilcoxon pair-wise comparisons with a significance level of $p \leq 0.05$. The analyses considered the main effects of and interactions between the variable factors of dose, time, and particle type. The data were analyzed for deviations from the assumptions of ANOVA; no outliers were identified via box plots.

RESULTS

Animal weight

Animal weight was unaffected by intratracheal instillation of the three tested TiO₂ forms compared to controls (data not shown). For animals exhibiting inflammation, there was no correlation between weight change following instillation and subsequent degree of inflammation post exposure (data not shown).

Toxic effects as determined with BALF

Total cell counts in BALF were similar between control and TiO₂ exposed animals, regardless of the dose or TiO₂ form (data not shown). While a dose-response for the percentage of BALF neutrophils was not found following exposure to the spherical TiO₂ nanoparticles, TiO₂-P25 and TiO₂-A, exposure to TiO₂-NB resulted in a significantly increased percent of BALF neutrophils 1-day post exposure at the highest dose (200 µg) compared to the control (Figure 2). However, by 7 days post exposure, BALF neutrophils had returned to control values.

While not resulting in toxicity as reflected by BALF, TiO₂ inclusions were seen in BAL macrophages of TiO₂-P25 and TiO₂-A exposed rats (Figure 3), suggesting that the nanospheres reached the deep lung. The percentage of macrophages containing visible inclusions ranged from approximately 0.5 to 20 percent in animals exposed to TiO₂-P25 or TiO₂-A (Figure 4, top vs bottom panels, respectively) remaining fairly stable from 1 to 7 days post exposure (Figure 4, left panels vs. right panels, respectively). While TiO₂-NB inclusions were found in BAL macrophages (Figure 3), the percentage of inclusions was not quantified because it was very difficult to consistently identify the delicate, fibrillar TiO₂-

NB structures with Brightfield microscopy; however, retention by macrophages was confirmed by TEM(Figure 5).

Total protein and LDH assays showed no significant differences between control and exposed animals (data not shown). Statistical comparisons of percent PMN and total cell data from animals exposed to 200 µg/250 µL of TiO₂ in PBS, sterile saline, and DM also showed no significant differences due to suspension media (data not shown).

Changes in the lung parenchyma

At 1 day post exposure, animals instilled with 200 µg of TiO₂ exhibited focal centriacinar bronchiolitis/alveolitis often in regions near visible particle agglomerates, or particle-laden macrophages in the air spaces (Figure 6). Qualitative assessment suggested TiO₂-NB caused a greater effect than either of the spherical TiO₂ nanoparticles. This is especially evident in the regions surrounding blood vessels, where heavy inflammatory infiltrates can be seen entering the airspaces (Figure 7, left). By day 7, inflammation appeared to decrease in animals exposed to TiO₂-P25 or TiO₂-A even though particles were still present in the alveoli. However, inflammatory infiltrates, and macrophages in the air spaces were abundantly visible at 7 days, in animals exposed to TiO₂-NB (Figure 7, right).

Lung burden of TiO₂ by ICP

When normalized to lung lobe mass, the cranial lobe was found to have less of all three TiO₂ forms compared to the caudal lobe at both 1 and 7 days post-exposure (Figure 6, top panel vs. bottom panel). These results reflect what was shown by microscopy; the lung retained TiO₂ nanoparticles 7 days after instillation. We feel confident about these results as over a detected concentration range of 0.06 to 0.35 µg TiO₂/L the correlation between ICP-MS and ICP-OES results was very high ($R^2 > 0.99$) with a linear slope within 6% of a one-to-one line.

DISCUSSION

The goal of this study was to assess the *in vivo* lung toxicity of nano-scale TiO₂ and determine a LOEL using three well characterized, highly dispersed TiO₂ forms. The three different forms of TiO₂ (anatase/rutile P25 spheres (TiO₂-P25), pure anatase spheres (TiO₂-A), and anatase nanobelts (TiO₂-NB)) were used to determine whether physico-chemical characteristics correlated to particle retention and/or inflammation. Instillation, rather than inhalation, was chosen as the route of exposure to precisely control dosimetry for LOEL determination. Despite alveolar macrophage TiO₂ inclusions being found for all three TiO₂ forms, only animals exposed to TiO₂-nanobelts (NB; highest dose of 200 µg) had a significantly greater percentage of neutrophils in BALF than control animals 1day post exposure; however, this resolved by 7days post-exposure. Due to lack of toxic response, a definite LOEL could not be determined for the TiO₂-P25 and TiO₂-A nanoparticles, although doses used are likely to be within a range of a worse-case exposure scenario in an occupational or environmental setting.

When taken with the sum of nano-scale TiO₂ research, results from this study mirror previous findings that not all TiO₂ engineered nanomaterials are equal in their ability to

elicit toxicity. Many *in vivo* TiO₂ studies to date 1) present incomplete particle characterization; 2) use questionable suspension media for intratracheal instillation procedures; 3) compare particles with many inherent differences; and/or 4) do not provide dosimetric measurements when correlating pulmonary toxicity and particle exposure. This study is unique in its careful attempt to control for these potentially confounding issues. Results from this study do not show a correlation between nanoparticle persistence and inflammation for spherical anatase-containing TiO₂, which is contrary to other studies suggesting that anatase TiO₂ is pathogenic (Warheit, Webb, Reed, et al. 2007; Zhang J 2012). TiO₂-A had the largest surface area to mass ratio (173 m²/g) of the three particles, and surface area has been correlated to increased inflammation in previous instillation (Oberdorster 2000; Oberdorster et al. 1992; Renwick et al. 2004; Saber, Jensen, et al. 2012) and inhalation studies (Bermudez et al. 2004; Oberdorster, Ferin, and Lehnert 1994).

Although TiO₂-P25 and TiO₂-A are clearly retained, this does not seem to influence neutrophilia. The single exposure may have insufficiently taxed the phagocytic defenses of the lung, or at these doses, TiO₂-P25 and TiO₂-A may not have reached the threshold surface area for eliciting inflammatory responses (Saber, Jacobsen, et al. 2012; Stoeger et al. 2006). Compared to the spherical TiO₂ nanoparticles, TiO₂-NB is an order of magnitude larger, fibrillar, and thus, has a high-aspect ratio. Research with asbestos and other high-aspect ratio nanoparticles shows impeded clearance from the lungs after inhalation exposure leading to the pathogenesis of diseases such as pulmonary fibrosis and mesothelioma (Bonner 2010). At present, there is evidence that high-aspect ratio TiO₂-NB are relatively more pathogenic, and may elicit "frustrated phagocytosis" by macrophages, lysosomal disruption, and impaired lung clearance (Donaldson et al. 2011; Hamilton et al. 2009). Indeed, long-term studies show a link between particle retention and pulmonary pathology (Bermudez et al. 2004; Oberdorster, Ferin, and Lehnert 1994).

Though relatively high bolus doses of TiO₂ (NIOSH recommends an average time-weighted concentration = 0.3 mg/m³ during a 40-hr work week) were used in this study and delivered by intratracheal instillation, it is important to understand that this approach does not truly mimic real-world exposure conditions or deposition patterns in the lung that would occur via inhalation. Intratracheal instillation was chosen to simply evaluate how the physico-chemical particle characteristics and retention of a precise dose of TiO₂ nanoparticles affects the lung. Instilled doses were chosen based on previous research showing significant acute pulmonary inflammation after exposure to ≤1 mg/kg of TiO₂ (Nemmar, Melghit, and Ali 2008; Rehn et al. 2003; Renwick et al. 2004; Sager, Kommineni, and Castranova 2008; Warheit, Webb, Reed, et al. 2007). We attempted to discern a LOEL using doses ranging from 0–0.5 mg/kg of bodyweight, and found the chosen dose range was not appropriate for all the TiO₂ forms. Though we were unable to determine a LOEL for the spherical TiO₂ forms tested, we did find an inflammatory LOEL of 0.5 mg/kg (200 µg) for TiO₂-NB, at 1-day post instillation using significant increase in BALF neutrophils as the toxic endpoint. To establish a LOEL for TiO₂-P25 and TiO₂-A, higher doses should be more informative. Further, responses following more realistic inhalation exposures should be evaluated with all three of these TiO₂ types in order to derive other lowest and/or no-effect levels for purposes of risk characterization and further development of exposure standards. This is especially

vital with the emerging nanotechnology industry and growing potential for human and environmental exposures due to commercial use.

It is generally accepted that dispersants and surfactants form weak associations with the nanoparticles, essentially adsorbing to them and forming dynamic, biomolecular “coronas” (Bihari et al. 2008; Ji et al. 2010; Nel et al. 2009; Pegueroles et al. 2012; Ruh et al. 2012; Sager et al. 2007; Shi et al. 2012; Walczyk et al. 2010; Xia et al. 2012). The coronas are largely determined by the types of nanoparticles and proteins present, and the length of time that they interact. Shi et al. reported that TiO₂ nanobelts with small diameters (~20 nm) adsorb less serum albumin (2012), a primary component of the Porter dispersion media we used, than nanobelts with large diameters (~200 nm). The inflammatory response elicited by the 10 nm TiO₂-NB in our study may have resulted from relatively greater “visibility” of TiO₂-NB in the lung than TiO₂-P5 and TiO₂-A. Indeed, according to Walczyk et al., naked nanoparticles have higher nonspecific affinities for the cell surface than coronated nanoparticles (2010). According to previous studies, surface chemistry (Warheit, Webb, Colvin, et al. 2007; Warheit, Webb, Reed, et al. 2007) and contact area (Hsiao and Huang 2011) between a nanoparticle and a single cell may actually be more important to toxicity than total specific surface area. TiO₂-NB were also found stuck in alveolar septa (Figure 10, left and middle). Still, ICP-MS showed less TiO₂-NB in the lung lobes than TiO₂-P25 or TiO₂-A. This may be due to the relatively higher aerodynamic diameter of TiO₂-NB, which could cause it to deposit higher in the respiratory tract and clear via mucociliary elimination (Figure 10, right).

Based on our results, we believe that future studies should focus on dose metrics to aid in streamlining engineered nanomaterials hazard identification and risk assessments. Nano-scale hyperspectral imaging would allow for accurate histological quantitation of TiO₂ without the complications of tissue digestion or interference of fluorescent/radio-labels.

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ABBREVIATIONS

BALF	Bronchoalveolar Lavage Fluid
BET	Bunauer, Emmett, & Teller
DM	Dispersion Media
DPPC	Dipalmitoylphosphatidylcholine
DSPC	Distearoylphosphatidylcholine
ENM	Engineered Nanomaterial
FLAR	Flame Aerosol Reactor
ICP-MS	Inductively-Coupled Mass Spectrometry
ICP-OES	Inductively-Coupled Optical Emission Spectrometry
IACUC	Institutional Animal Care and Use Committee
IARC	International Agency for Research on Cancer
LDH	Lactate Dehydrogenase
LOEL	Lowest Observed Effect Level
RSA	Rat Serum Albumin
SEM	Scanning Electron Microscopy
TiO₂	Titanium Dioxide
TEM	Transmission Electron Microscopy

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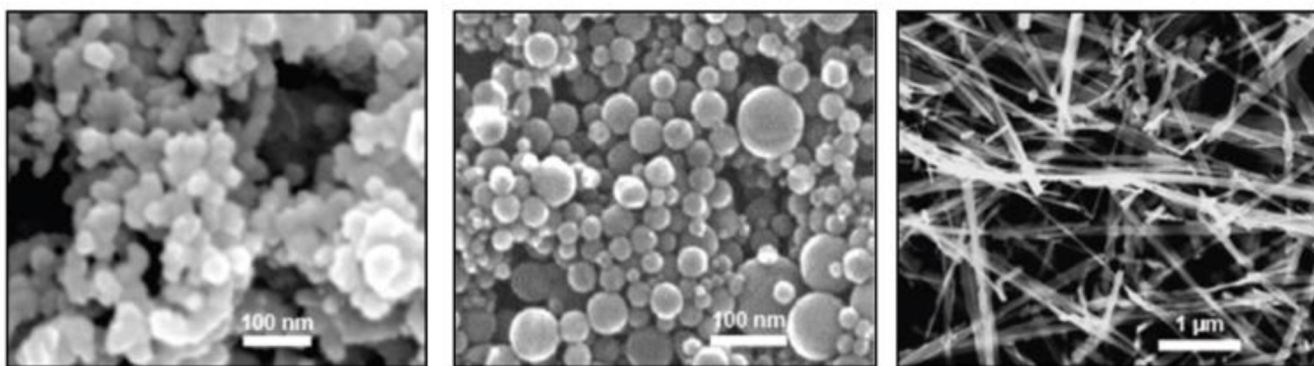


Figure 1. SEM images of all studied nanomaterials in pristine form

From left to right: TiO₂-P25, TiO₂-A, and TiO₂-NB. Spherical particle types, TiO₂-P25 and TiO₂-A, averaged approximately 26 nm in diameter. TiO₂-NB averaged 7000 nm in length and 200 nm in width, and individual nanobelts were approximately 10 nm thick. Scale indicated by the white bars. Reproduced with permission from *Environmental Health Perspectives*.

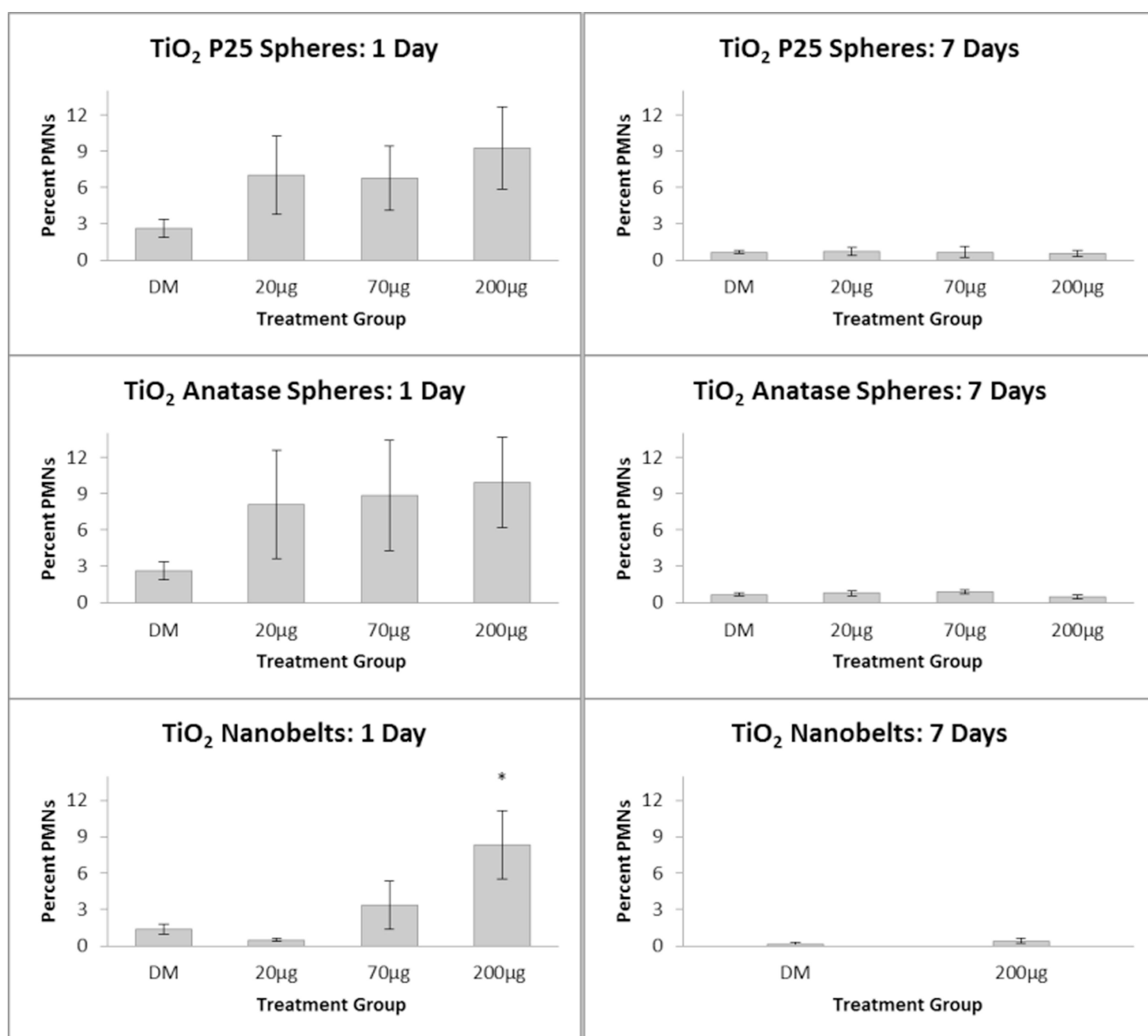


Figure 2. Stimulation of neutrophilic inflammation in the lungs of rats by TiO₂ engineered nanomaterials

Percentages of neutrophils (PMNs) in BALF 1 day (left panels) and 7 days (right panels) after IT exposure to P25 nanospheres (top), anatase nanospheres (middle), or anatase nanobelts (bottom). *P<0.05 compared to DM-exposed controls.

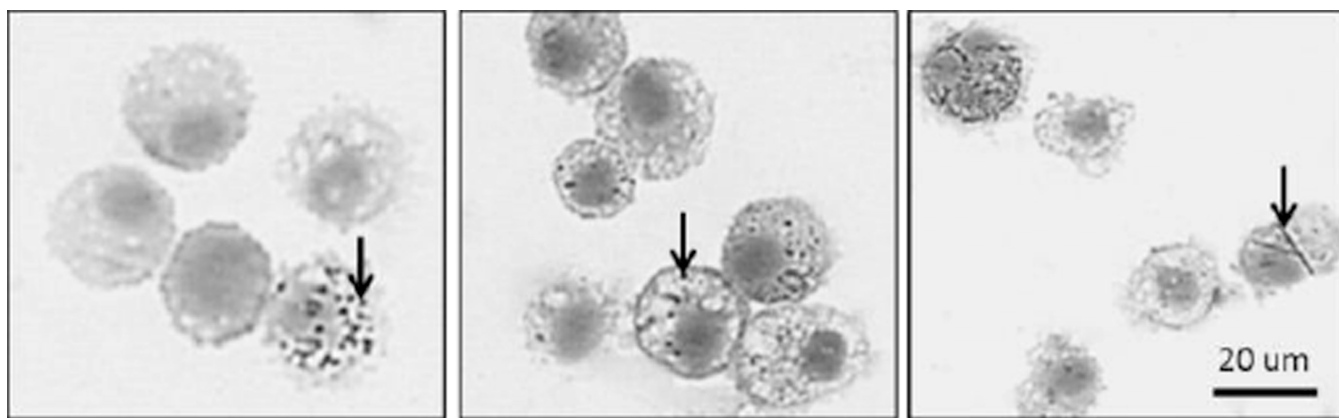


Figure 3. Instilled TiO₂-A causes moderate but sustained particle loading in alveolar macrophages

Cells recovered from BALF at 7 days post exposure to TiO₂-P25 (left), TiO₂-A (middle) and TiO₂-NB (right) by intratracheal instillation. Panels are Phase Contrast microscopy images of representative cells from rats given a single dose of TiO₂. BAL cells were stained with Diff Qwik®. Arrows indicate TiO₂ nanoparticles. Scale indicated by black bar.

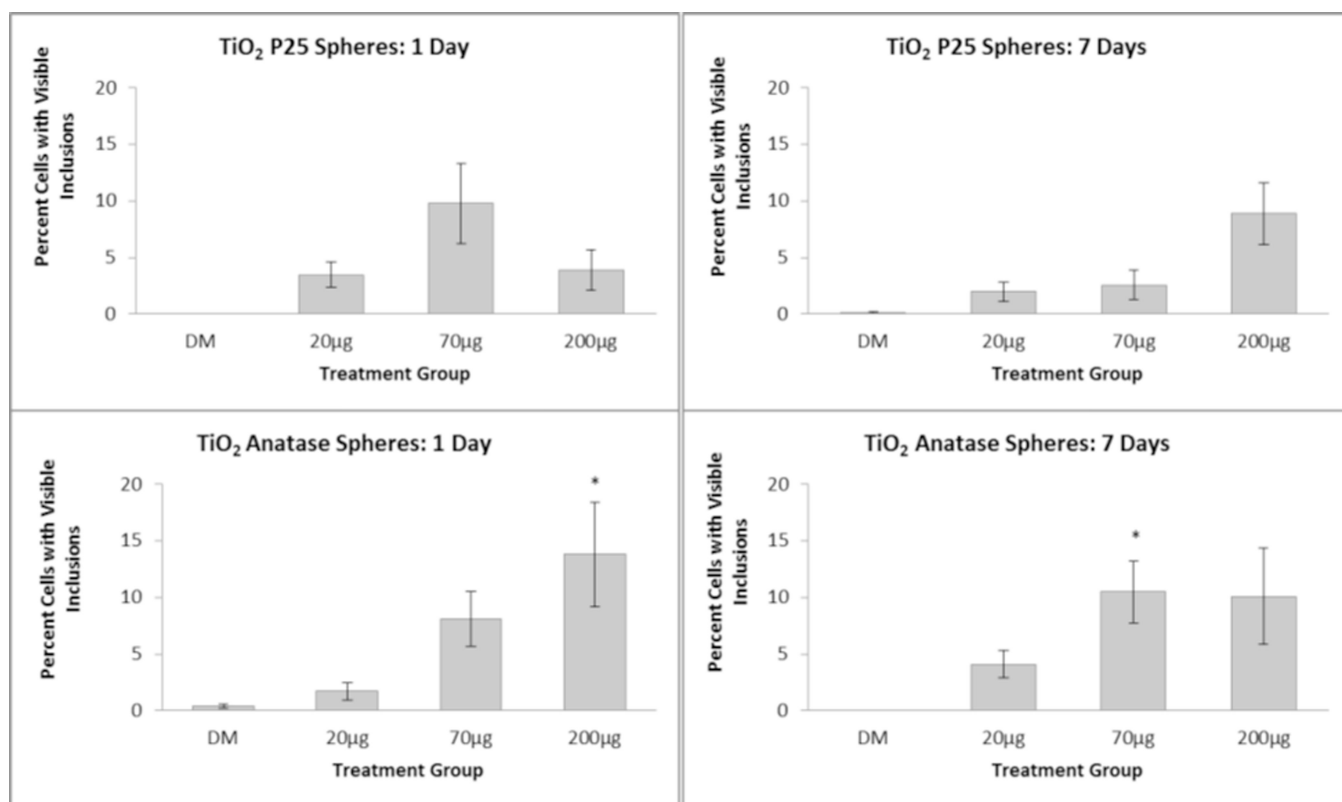


Figure 4. TiO₂ engineered nanomaterials uptake by macrophages in the lungs

Percentages of macrophages in BALF with visible particle inclusions 1 day (left panels) and 7 days (right panels) after intratracheal instillation exposure to TiO₂-P25 nanospheres (top) or TiO₂-A nanospheres (bottom). *P<0.05 compared to DM-exposed controls.

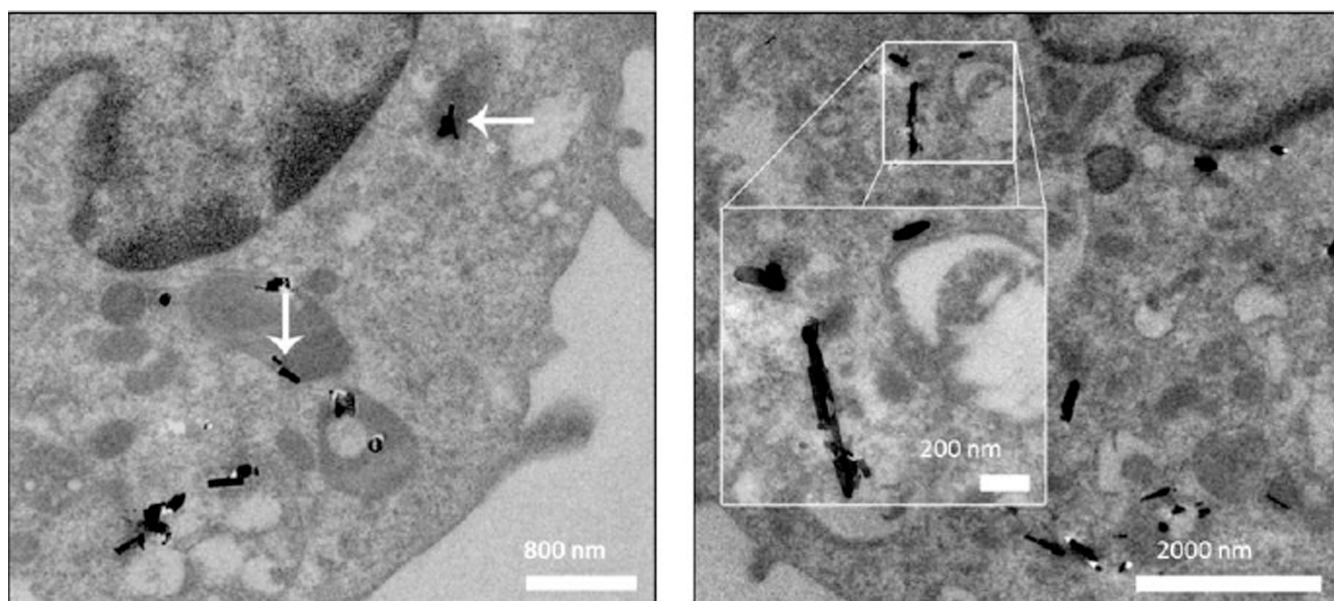


Figure 5. TEM images of TiO₂ nanobelts (TiO₂-NB) in alveolar macrophages
Cells recovered from BALF at 1 (left) and 7 (right) days post instillation. Panels are images of representative cells from rats given 200 µg TiO₂-NB. White arrows indicate TiO₂-NB aggregates. Scale indicated by the white bars.

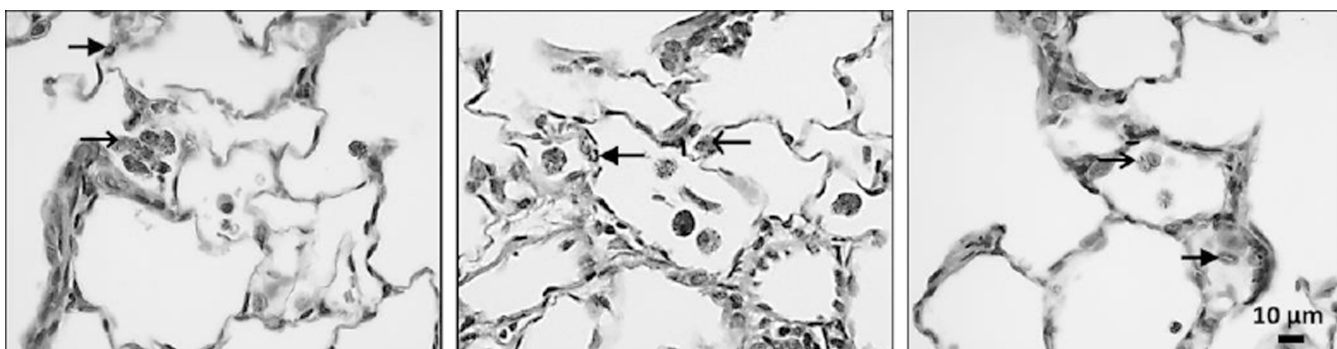


Figure 6. TiO₂ causes inflammatory infiltrates in the left lung at 1 day post exposure
Histopathological findings from exposure to 200 μg/250 μL of TiO₂-P25 (left), TiO₂-A (middle) and TiO₂-NB (right) by intratracheal instillation. Panels are Brightfield microscopy images of representative lung tissues from rats given a single dose of TiO₂. Tissues were stained with hematoxylin and eosin. Closed arrow = inflammatory cell infiltrate; Open arrow = particle laden cells; TB = terminal bronchiole. Scale indicated by black bar.

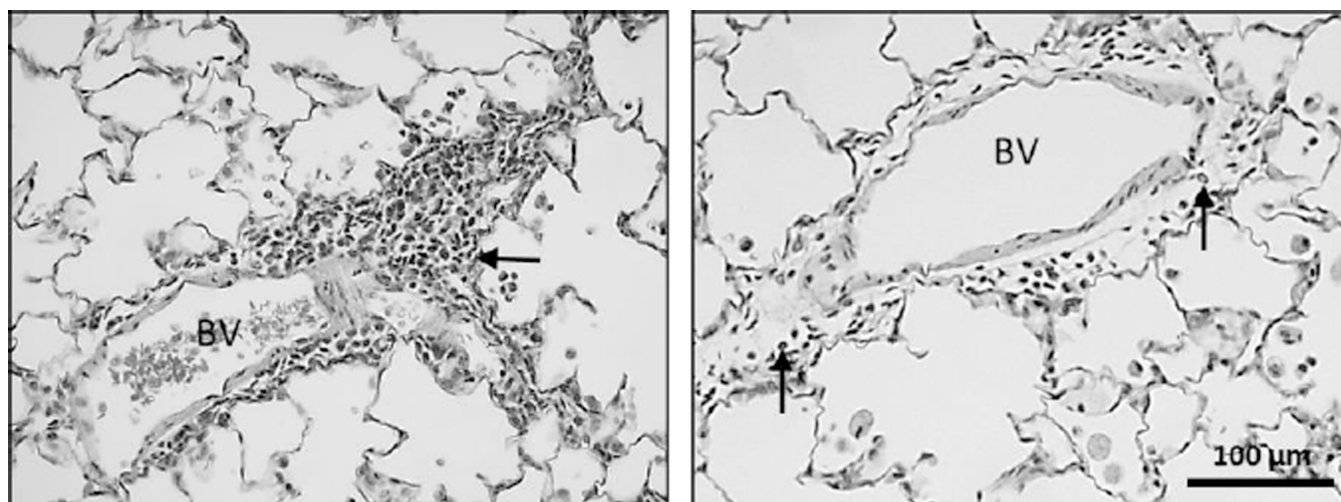


Figure 7. Inflammatory cells can be seen at 1 and 7 days post exposure to TiO₂-NB
Histopathological findings from exposure to 200 μg/250 μL of TiO₂-NB by intratracheal instillation. Panels are Brightfield microscopy images of representative lung tissues from rats given a single dose of TiO₂. Tissues were stained with hematoxylin and eosin. Closed arrow = mixed inflammatory cell infiltrate; BV = blood vessel. Scale indicated by black bar.

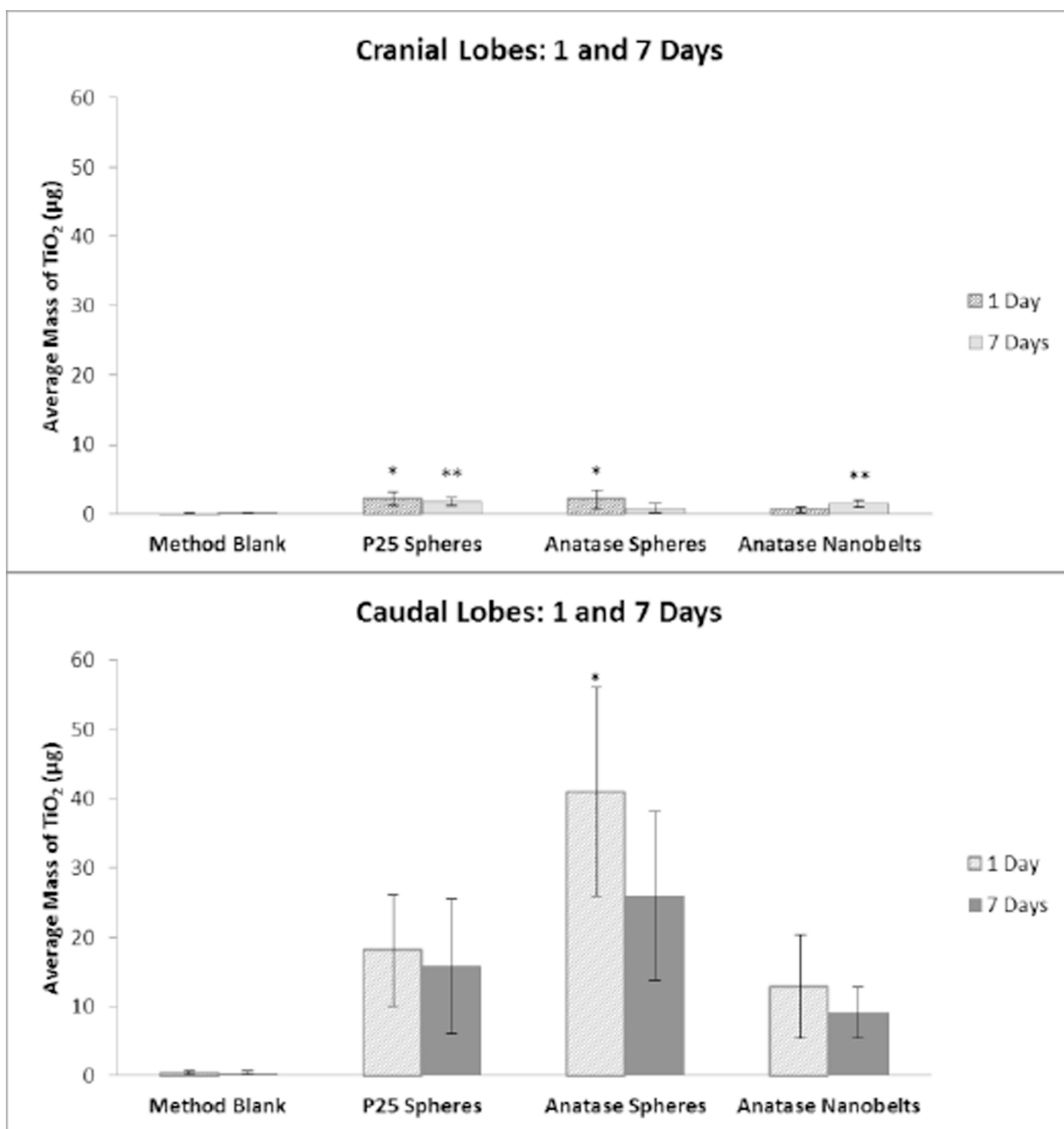


Figure 8. TiO₂ lung burden in the right cranial and caudal lobes

Average mass of TiO₂ observed in the cranial (top panel) and caudal (bottom panel) lung lobes 1 day and 7 days after intratracheal instillation exposure to TiO₂-P25 nanospheres, TiO₂-A nanospheres, or TiO₂-NB. *,**P<0.05 compared to method blank (control) from 1 day and 7 days, respectively.

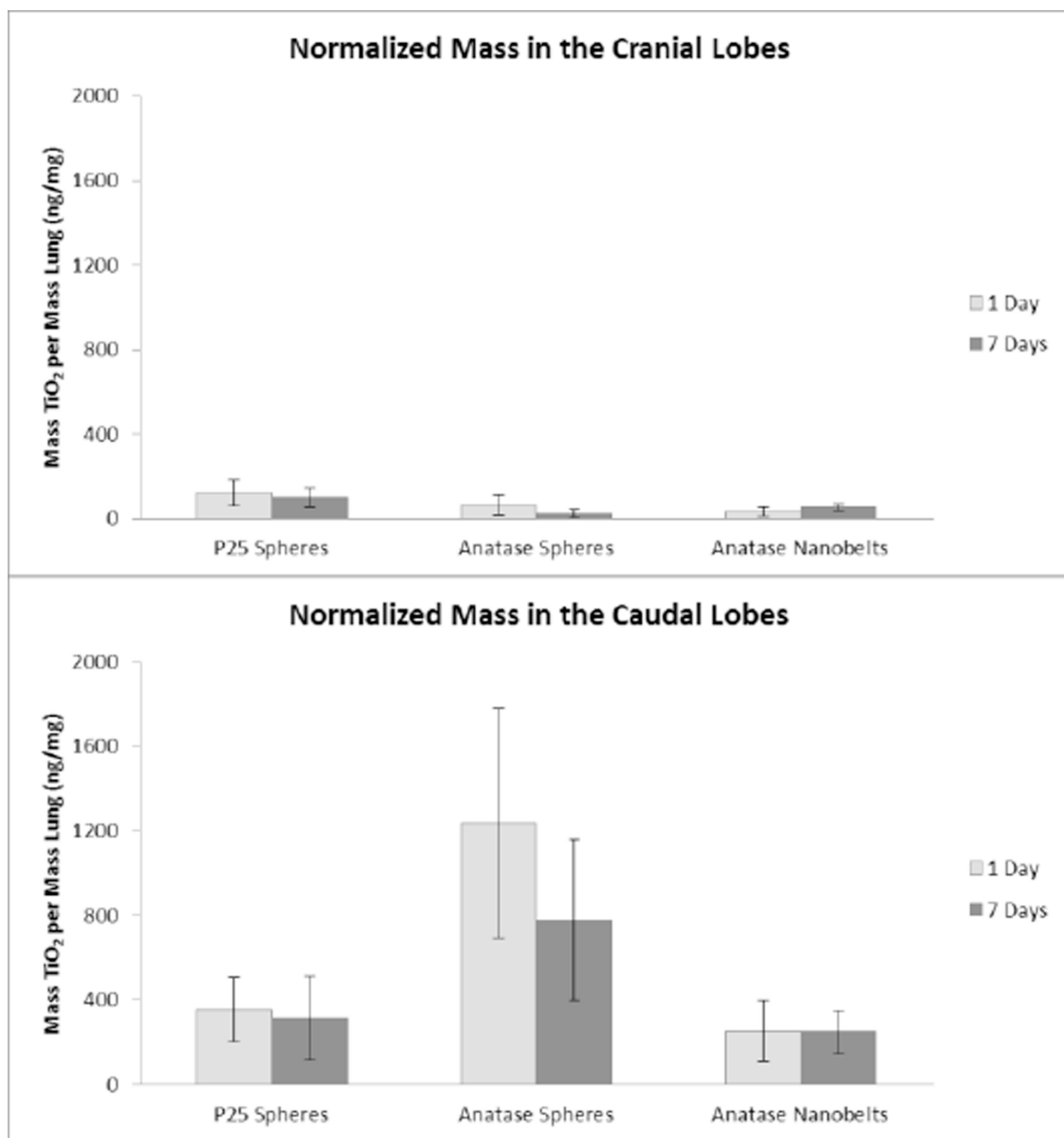


Figure 9. TiO_2 lung burden in the right cranial and caudal lobes normalized by lung mass
Normalized mass of TiO_2 observed in the cranial (top) and caudal (bottom) lung lobes 1 and 7 days after a single IT exposure.

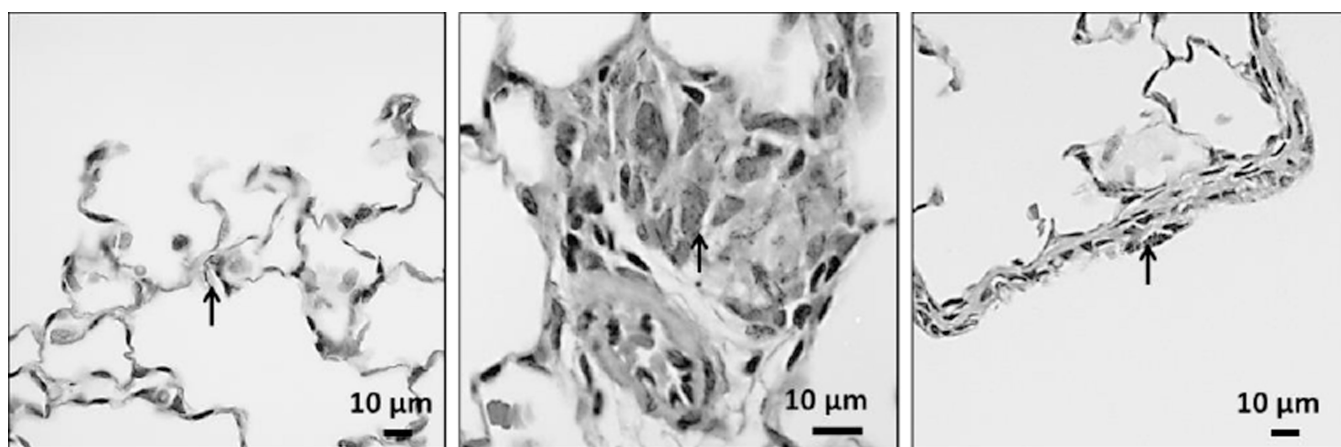


Figure 10. Deep lung penetration of TiO₂ nanobelts at 1 day post exposure

Histopathological findings from exposure to 200 µg/250 µL of TiO₂-NB by intratracheal instillation. Panels are Brightfield microscopy images of representative lung tissues from rats given a single dose of TiO₂. Tissues were stained with hematoxylin and eosin. Open arrow = TiO₂-NB aggregates. Scale indicated by black bar.