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RESEARCH ARTICLE

Changing the dose metric for inhalation toxicity studies: Short-term study in rats with engineered aerosolized amorphous silica nanoparticles

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Abstract

Inhalation toxicity and exposure assessment studies for nonfibrous particulates have traditionally been conducted using particle mass measurements as the preferred dose metric (i.e., mg or μg/m^3). However, currently there is a debate regarding the appropriate dose metric for nanoparticle exposure assessment studies in the workplace. The objectives of this study were to characterize aerosol exposures and toxicity in rats of freshly generated amorphous silica (AS) nanoparticles using particle number dose metrics (3.7×10^7 or 1.8×10^8 particles/cm^3) for 1- or 3-day exposures. In addition, the role of particle size (d_{50} = 37 or 83 nm) on pulmonary toxicity and genotoxicity endpoints was assessed at several postexposure time points. A nanoparticle reactor capable of producing, *de novo* synthesized, aerosolized amorphous silica nanoparticles for inhalation toxicity studies was developed for this study. SiO_2 aerosol nanoparticle synthesis occurred via thermal decomposition of tetraethylorthosilicate (TEOS). The reactor was designed to produce aerosolized nanoparticles at two different particle size ranges, namely d_{50} = ~30 nm and d_{50} = ~80 nm; at particle concentrations ranging from 10^7 to 10^8 particles/cm^3. AS particle aerosol concentrations were consistently generated by the reactor. One- or 3-day aerosol exposures produced no significant pulmonary inflammatory, genotoxic, or adverse lung histopathological effects in rats exposed to very high particle numbers corresponding to a range of mass concentrations (1.8 or 86 mg/m^3). Although the present study was a short-term effort, the methodology described herein can be utilized for longer-term inhalation toxicity studies in rats such as 28-day or 90-day studies. The expansion of the concept to subchronic studies is practical, due, in part, to the consistency of the nanoparticle generation method.

Keywords: *Amorphous silica particles; dose metric; inhalation toxicity studies; lungs; nanoparticles; nanotoxicity studies; particle aerosols; particle numbers; particle size distribution*

Introduction

Nanotechnology involves the design and manipulation of materials at the nanoscale size range, concomitant with the corresponding development of products with versatile properties. The capacity for predicting occupational hazards or quantifying exposures to engineered nanomaterials for workers or consumers is limited at this time, and may require new methodologies to provide the necessary information for effective risk management. In addition, measuring the inherent toxicity of engineered nanomaterials is a new challenge in the field of toxicology (Oberdörster et al., 2005; Borm et al., 2006). Although methods for nanoparticle synthesis exist, few tools are available to produce well-characterized, monodispersed, aerosolized nanoparticles at relatively high concentrations. In a previous study, Ostraat et al. (2008) developed a nanoparticle reactor capable of producing aerosolized amorphous SiO_2 particles. This reactor was designed to produce aerosols of

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nanoparticles (d_{50} = 10–100 nm; [particle numbers] ~ 10^4–10^6 particles/cm^3); and the subsequent goal was to test in rats, a continuous, stable supply of nanoparticles contiguous with an exposure chamber.

This study was designed to assess the pulmonary toxicity in rats of inhaled engineered amorphous silica nanoparticles following short-term exposures. Concomitant with this inhalation study, a genetic toxicology assessment of micronuclei induction was implemented to evaluate the potential for amorphous silica (AS) nanoparticles to induce increases in the frequency of micronucleated reticulocytes (MN-RETs) in peripheral blood cells. An increase in this frequency relative to control samples would indicate an induction of chromosome and/or spindle damage in erythroblasts. This endpoint was included in order to optimize the utilization of animals, and to add data from an *in vivo* MN assay to the existing database of nano(genotoxicity) findings using conventional methods. To our best knowledge, currently there are no publicly available databases on analyses of *in vivo* micronuclei in rats after exposure to a variety of nanomaterials (Landsiedel et al., 2009; Gonzales et al., 2008).

Most inhalation toxicity studies with nonfibrous particulates have been conducted in rats or mice using gravimetric dose metrics (e.g., measured concentrations of mg/m^3) as exposure measures (Warheit et al., 1997). It has also been suggested that particle surface area should be considered as a dose metric (Warheit et al., 2007a). Here, we attempted to generate and measure aerosols via particle numbers and extrapolate the exposure concentrations to gravimetric units. A pulmonary bioassay in rats has previously been developed to assess the effects of particle exposures on the lungs (Warheit et al., 1997).

Methods

A schematic diagram of the SiO_2 aerosol nanoparticle reactor is shown in Figure 1. This nanosilica particle-type was utilized as a prototype for generating freshly generated, monodispersed amorphous silica particle-types. The reactor consists of a N_2 gas flow attached to a precursor evaporator. The precursor (tetraethylorthosilicate [TEOS], $Si(OC_2H_5)_4$) is contained within this controlled temperature evaporator. As the N_2 gas flows through the evaporator, it mixes with the TEOS vapor from the TEOS liquid surface and delivers it to a quartz tube reactor within a furnace. In the tube furnace, the TEOS vapor undergoes thermal decomposition. Upon the onset of homogeneous nucleation at 850°C, nanoparticles are produced using the "seeded growth" methodology. This refers to the method of nanoparticle synthesis where nucleation sites are created from precursor vapors at high temperatures and pressures. Growth of each nucleation site is maintained with precursor concentration via N_2 gas flow and furnace temperature (Ostraat et al., 2008).

The generated SiO_2 aerosol nanoparticles exit the furnace, cool, and enter a Kr^{85} aerosol neutralizer. The neutralizer charges the particles with beta particles. Once the aerosol acquires a known charge distribution, a size distribution of the generated particles was plotted. The aerosolized nanoparticles then flowed through the characterization instrumentation, and subsequently were sampled using a long differential mobility analyzer (long DMA), a nano differential mobility analyzer (nano DMA), a condensation nucleus counter (CNC), and an aerosol electrometer (AE). The long DMA (TSI model 3081) is suitable for classifying aerosol particles with a mobility diameter between 20 and 250 nm. The nano DMA (TSI model 3085) was used to classify aerosol particles between 3 and 50 nm. Once the particles were classified according to their size, the particle number concentration was measured with a CNC (TSI model 3025A) for low particle concentrations (<10^5 particles/cm^3) or an AE (TSI model 3068A) for high particle concentrations (10^3–10^9 particles/cm^3) (Ostraat et al., 2008).

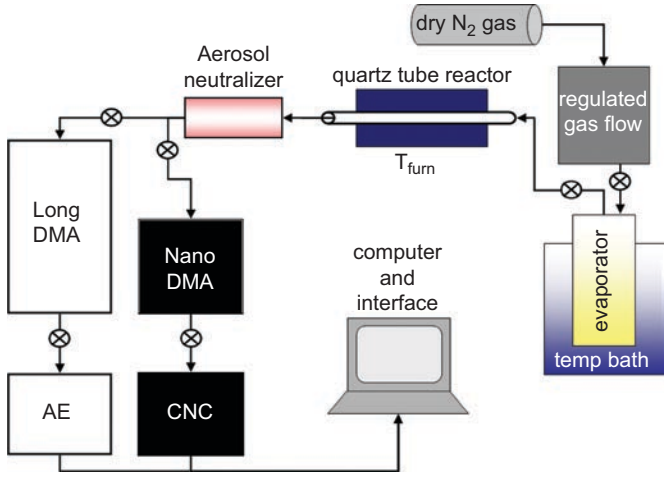


Figure 1. Schematic diagram of the aerosol nanoparticle reactor with characterization instrumentation. TEOS was pyrolyzed to generate SiO_2 nanoparticles that are charged with an aerosol neutralizer, classified with a long or nano DMA, and measured for particle concentration with a CNC or AE. Figure adapted from Ostraat et al. (2008).

General experimental design

The fundamental features of this pulmonary bioassay are dose-response evaluations and time-course assessments to determine the sustainability of any observed effect (Warheit et al., 2007b). Thus, the major biological endpoints of this study were the following: (1) time course and dose-response intensity of pulmonary inflammation and cytotoxicity; (2) histopathological evaluation of lung tissue; and (3) genetic toxicology evaluation of micronuclei in blood.

Groups of male Crl:CD (SD)IGS BR rats (Charles River Laboratories, Raleigh, NC) were used in this study. The rats were approximately 8 weeks old at study start (mean weights in the range of 210–280 g). All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee. The animal program

is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

For the bronchoalveolar lavage studies, groups of rats (5 rats/group/dose/time point) were exposed nose-only to aerosols of amorphous silica nanoparticles (d_{50} = 37 or 83 nm) at concentrations ranging from 3.1×10^7 to 1.8×10^8 particles/cm^3 or to room air for 1 or 3 days (6 h/day) (see Table 1). The lungs of phosphate-buffered saline (PBS)-instilled and particle-exposed rats were evaluated by bronchoalveolar (BAL) fluid analyses at 24 h, 1 week, 1 month, and by histopathology at 2 months post exposure.

For the lung tissue studies, additional groups of AS or sham-exposed animals (4 rats/group/high dose/time period) were evaluated at the 2-month postexposure time period. These studies consisted of histopathological evaluations of the lower respiratory tract (Warheit et al., 2007b).

For the micronucleus analyses, samples were collected 24 h post exposure for the 1- and 3-day exposures with both particle sizes and for the 3-day exposures for the sham controls.

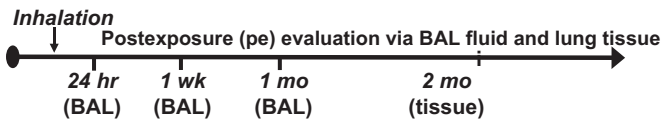
The lungs of sham- and particulate-exposed rats were lavaged with a phosphate-buffered saline (PBS) solution as described previously. Methodologies for cell counts, differentials, and pulmonary biomarkers in lavaged fluids were conducted as previously described (Warheit et al., 1997). Biochemical assays for lactate dehydrogenase (LDH), microprotein, and alkaline phosphatase were performed on BAL fluids using a Roche Diagnostics (BMC)/Hitachi 717 clinical chemistry analyzer using Roche Diagnostics (BMC)/Hitachi reagents. Lactate dehydrogenase is a cytoplasmic enzyme and is used as an indicator of cell injury. Alkaline phosphatase 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 microprotein 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.

Groups of nanoparticle-exposed rats and corresponding controls were infused with fixative at 2 months post exposure. The methodology for histopathology of lung tissues has been previously described in detail (Warheit et al., 2007b).

Table 1. General experimental design.

Exposure Groups

- Group 1 (3-day exposure)
  Sham (5 rats/group)
  Particle-exposed (5 rats/group)
  Targeted particle sizes = 35 nm and 80 nm
- Group 2 (1-day exposure)
  Sham (5 rats/group)
  Particle-exposed (5 rats/group)
  Targeted particle sizes = 35 nm and 80 nm



Analysis of in vivo micronucleus induction was performed as previously described (Torous et al., 2000). Peripheral blood samples were extracted via the orbital sinus from the exposed groups of rats at 24 h post exposure for the 1- and 3-day exposures. Samples at later time points—1 week and 1 and 2 months post exposure—were excluded from the analyses due to the short-term exposure durations and the quick elimination of any induced micronucleus (MN) post exposure (Heddle et al., 1983). Approximately 2 to 3 drops (i.e., 60–120 µl) of blood were collected from the orbital sinus of each animal directly into a labeled microcentrifuge tube containing 350 µl Solution B (anticoagulant) found in the In Vivo MicroFlow^{Plus} Rat Micronucleus assay kit (Litron Laboratories, Rochester, NY). The tubes were capped and inverted several times to mix the blood with Solution B. The samples (approximately 180 µl of blood/Solution B mixture) were fixed in 2 ml ultracold Solution A (methanol), found in the In Vivo MicroFlow^{Plus} Rat Micronucleus assay kit, and stored below −75°C until labeled, washed, and analyzed according to the supplier's instructions (Litton MicroFlow^{Plus} (Rat Blood) Instruction Manual, Version 090203).

Using a flow cytometer, at least 20,000 reticulocytes were analyzed per blood sample for both toxicity and the induction of micronuclei. The samples were analyzed with a BD FACSCalibur™ Flow Cytometer using BD CellQuest Pro Software, and evaluated using the In Vivo MicroFlow^{Plus} Rat Blood micronucleus assay kit. The frequency of micronucleated reticulocytes (%MN-RETs) was used as a measure of induction of aneugenic or clastogenic alterations by the test substance. Toxicity was indicated by the frequency of immature erythrocytes (% reticulocytes [RETs]) among the total (RETs plus normochromatic erythrocytes [NCEs]). The 3-day sham sample was used as negative control, and the positive control biological standard provided in the assay kit was used as a methods positive control.

For statistical analyses, each of the experimental values was compared to their corresponding sham control values for each time point. A one-way analysis of variance (ANOVA) and Bartlett's test were calculated for each sampling time. When the F test from ANOVA was significant, the Dunnett's test was used to compare means from the control group to each of the groups exposed to particulates. Significance versus PBS controls was judged at the 0.05 probability level (Warheit et al., 2007b).

Results

The focus of this study was on the synthesis, physical characterization, and initial pulmonary toxicity assessments for nano-sized amorphous silica particle-types. Aerosols of the amorphous silica nanoparticle test materials of untreated, as-synthesized, amorphous SiO_2 nanoparticles, in two different size range populations (i.e., particle number distributions centered at d_{50} = 37 and d_{50} = 83 nm),

were generated. Each of the size populations was developed *in situ* and upstream of the inhalation exposures. Particle size distribution (PSD) plots emphasize the consistency and lack of particle aggregation carried through to the inhalation chamber over the 3-day period (Figure 2). The particles subsequently were agglomerated on a filter (Figure 3). The nanoparticles generated in this inhalation experiment were not placed in solvent, thus eliminating variability due to coagulation, aggregation, or agglomeration. The aerosolized nanoparticle populations were tested for stability over time. Background aerosol nanoparticle size distributions in the inhalation chamber demonstrated low background counts for nanoparticle aerosols (Figure 4).

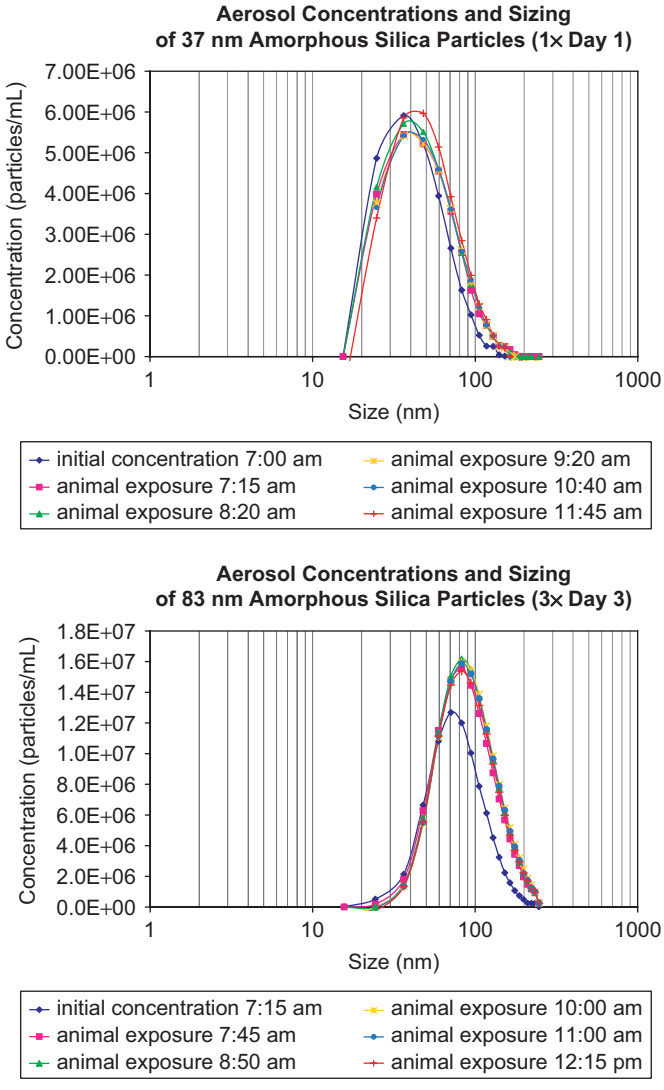


Figure 2. Aerosol nanoparticle size distributions for SiO_2 exposure in the inhalation chamber as a function of exposure time on day 1 (top) and day 3 (bottom) demonstrating aerosol stability. One measurement (initial exposure concentration) was made at the beginning of each exposure and five subsequent measurements were conducted during the 6-h exposure period. The top graph is a typical aerosol exposure run 1st day for the $d_{50} = 37$ nm particle generation experiment. The bottom graph is a typical exposure run on day 3 for the $d_{50} = 83$ nm particle exposure experiment. Particle sizing and counts were measured as dN/dlogDp.

Calculations of particle mass were conducted based on the particle number data and assuming the density of amorphous silica to be 2.2 g/cm³. It is assumed that the particle shape is a sphere for the mass concentration calculation.

$$\text{Volume of sphere} = \frac{4 * \pi * r^3}{3}$$

$$\text{Mass of particle} = \text{volume} \times \rho$$

$$\text{Concentration} = \left\{ \begin{matrix} \text{mass} \\ (\text{mg/particle}) \end{matrix} \right\} \times \left\{ \begin{matrix} \text{number of particles} \\ (\text{particle/m}^3) \end{matrix} \right\}$$

$$= \text{mg/m}^3$$

$$d_1 := 37\,\text{nm}, \quad r := d/2, \quad r_1 = 18.5\,\text{nm} \quad \rho = 2.2\,\text{g/cm}^3$$

$$\text{mass}_1 = 5.8 \times 10^{-14}\ \text{mg/particle}$$

$$\text{conc}_1 = 5.8 \times 10^{-14}\ \text{mg/particle} * 3.17 \times 10^{13}\ \text{particles/m}^3$$

$$= 1.8\,\text{mg/m}^3$$

Alternate method used for calculating gravimetric concentration:

$$d_2 := 83\,\text{nm}, \quad r := d/2, \quad r_2 = 41.5\,\text{nm} \quad \rho = 2.2\,\text{g/cm}^3$$

$$\text{mass}_2 = 6.587 \times 10^{-13}$$

$$\text{conc}_2 = 1.3 \times 10^8 \times \text{mass}_2$$

$$\text{conc}_2 = 8.562 \times 10^{-5}\,\text{mg/cm}^3 = 86\,\text{mg/m}^3$$

Accordingly, the mass concentrations were calculated to be 1.8 mg/m³ (1.8×10^{-6} mg/cm³) for the exposures to $d_{50} = 37$ nm amorphous silica particulates; and 86 mg/m³ (8.6×10^{-5}) for the exposures to the $d_{50} = 83$ nm amorphous silica particle-types.

Groups of rats were exposed nose-only 6 h/day for 1 or 3 consecutive days to aerosols of amorphous silica (AS) nanoparticles ($d_{50} = 37$ nm) at particle number concentrations averaging 3.1×10^7 particles/cm³. Additional groups were exposed for 1 or 3 consecutive days to aerosols of larger ($d_{50} = 83$ nm) AS nanoparticle-types at concentrations averaging 1.3×10^8 particles/cm³. The results demonstrated that amorphous SiO_2 nanoparticles were reproducibly and consistently generated and delivered to the inhalation chamber by the reactor. The reaction was stable on length scales from minutes to 3 days. Following exposures, no significant pulmonary inflammatory responses were measured in the lungs of AS nanoparticle or sham-exposed air controls at any postexposure time period (Figure 5). Likewise, no differences vs. controls were measured in lung lavage cytotoxicity parameters (BAL fluid LDH [Figure 5], microprotein [data not shown], or alkaline phosphatase values [data not shown] at any postexposure time point. Evaluation of lung tissues at 2 months post inhalation exposure demonstrated normal lung architecture, indicating no significant pulmonary impacts to following particle exposures (Figure 6).

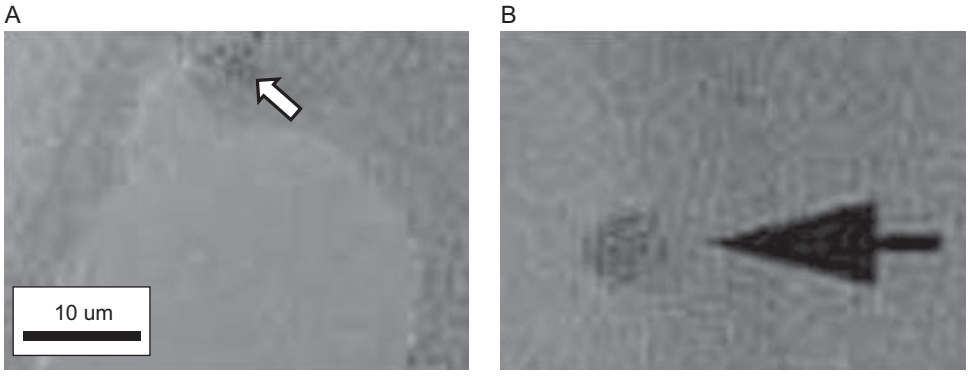


Figure 3. Transmission electron micrographs (TEM) of amorphous silica nanoparticles (arrows) generated by thermal decomposition of tetraethylorthosilicate. TEMs of (A) individual amorphous SiO_2 nanoparticles and (B) an agglomerate of amorphous SiO_2 nanoparticles.

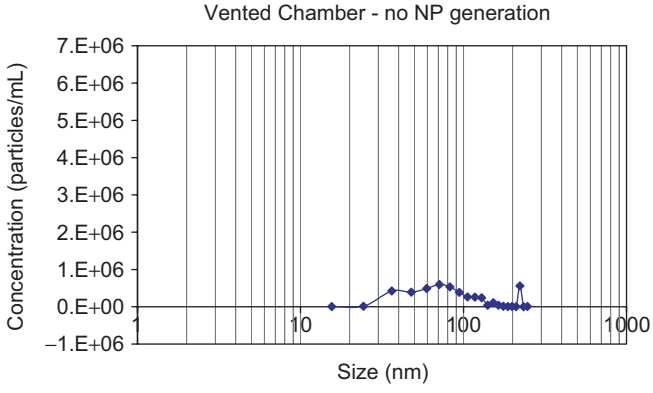


Figure 4. Background aerosol nanoparticle size distributions in the inhalation chamber demonstrated low background counts for the nanoparticle aerosol.

The *in vivo* micronucleus assay using rodent peripheral blood and analyzing micronuclei by flow cytometry has been shown to be applicable to both acute and chronic exposures of rats (Cammerer et al., 2007), and to be more sensitive than the conventional microscopic method (Witt et al., 2008). The results of the flow cytometry–based micronucleus analyses demonstrated that the AS exposures did not induce detectable levels of micronuclei. The slight decrease in the percent immature reticulocytes is considered a function of age of the animal and the hematopoietic cells rather than a compound-related effect (Table 2). Due to the apparent lack of previously published *in vivo* MN nanoparticle data, these results cannot be compared to any existing database.

Discussion

This study was designed to evaluate the pulmonary toxicity in rats inhaling freshly generated amorphous silica nanoparticles following short-term exposures. The engineered silica nanoparticles were utilized as prototypes for generating monodispersed nanoparticulates.

Particle inhalation toxicity and exposure assessment studies have traditionally been conducted using

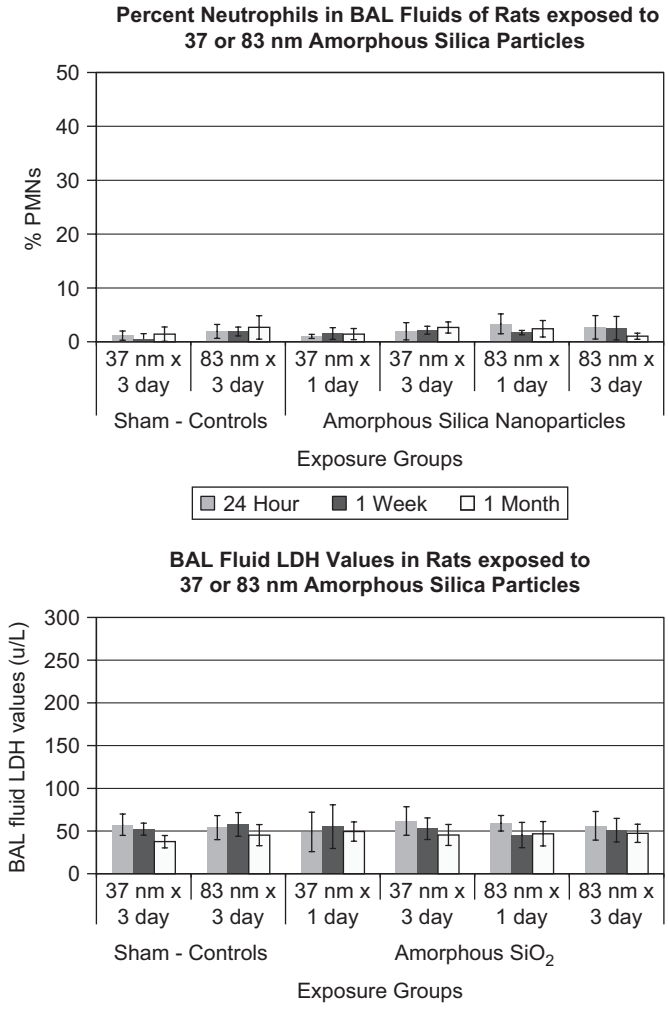


Figure 5. Assessments of pulmonary inflammation (top) and BAL fluid lactate dehydrogenase values (bottom) in rats exposed to aerosols of amorphous silica nanoparticles or to room air. Rats were exposed for 1 or 3 days, 6 h/day to 37- or 83-nm AS nanoparticles at aerosol concentrations of 3.1×10^7 or 1.8×10^8 particles/cm^3. Values given are mean percentages of neutrophils ± *SD* at 24 h, 1 week, and 1 month post exposure. No significant effects were measured in the lungs of nanoparticle-exposed rats when compared to sham controls at any measured time point.

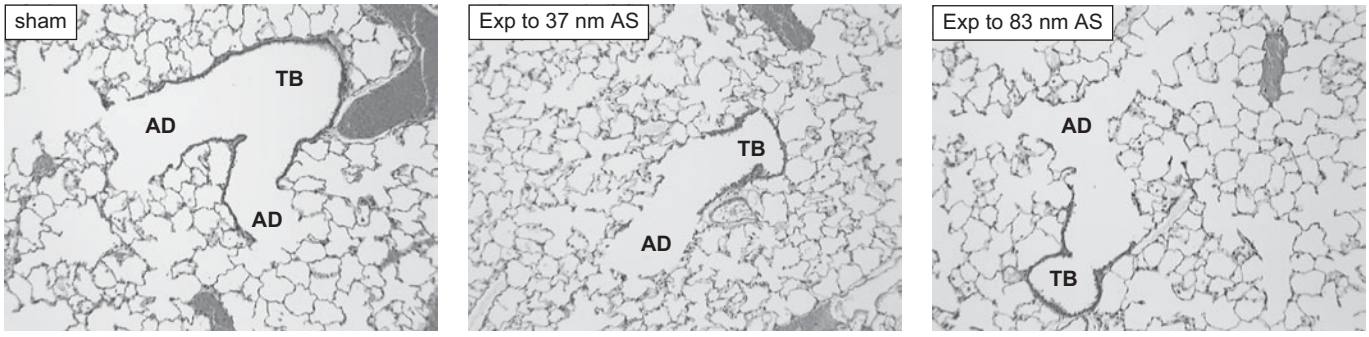


Figure 6. Tissue analyses of the rat lung exposed to 37- or 83-nm AS particles for 3 days at the 2-month postexposure time point showing no lung injury. Instead, the micrograph illustrates normal lung architecture. Exp = exposure; AS = amorphous silica; TB = terminal bronchiole; AD = alveolar duct.

Table 2. Micronucleus summary data.

Measurement at the 24-h postexposure time point	Amorphous silica (AS) 37-nm particles			Amorphous silica (AS) 83-nm particles		
	Solvent control, 3-day exposure	Experimental sample, 1-day exposure	Experimental sample, 3-day exposure	Solvent control, 3-day exposure	Experimental sample, 1-day exposure	Experimental sample, 3-day exposure
RETs (%)	4.07	4.37	3.36	4.30	4.52	4.09
	0.73 (5)	0.39 (4)	0.94 (5)	0.19 (5)	0.62 (4)	0.71 (4)
MN-RETs (%)	0.10	0.09	0.11	0.14	0.11	0.12
	0.03 (5)	0.01 (4)	0.01 (5)	0.08 (5)	0.03 (4)	0.03 (4)

Note. Data are mean (top) standard deviation (number of values included in calculation) (bottom).

particle mass or a gravimetric measurement as the dose metric (i.e., mg or μg/m^3) (Oberdörster et al., 2005; Borm et al., 2006). However, currently there is a serious debate regarding the appropriate dose metric for exposure assessment studies in the workplace for engineered nanoparticles. Particle number concentration has been suggested as a more appropriate exposure metric than mass, based upon limitations in conducting gravimetric sampling (Warheit et al., 2007a). Accordingly, a nanoparticle reactor capable of producing de novo synthesized, aerosolized amorphous silica nanoparticles for inhalation toxicity studies was developed for this study. SiO_2 aerosol nanoparticle synthesis occurred via thermal decomposition of tetraethylorthosilicate (TEOS). The reactor was designed to produce aerosolized nanoparticles at two different particle size ranges, namely d_{50} = ~30 nm and d_{50} = ~80 nm, and at particle concentrations ranging from 10^7 to 10^8 particles/cm^3. The specific objectives of this study were to characterize the exposures in particle number dose metrics, estimate the mass concentration, and assess the role of particle size on pulmonary toxicity for freshly generated amorphous silica nanoparticles in rats; with an added genotoxicity micronucleus endpoint in peripheral blood samples.

Short-term inhalation exposures to high concentrations of particles (10^8 particles/cm^3) did not provoke inflammatory or genotoxic responses in the lungs of rats. The high particle numbers corresponded to a range of mass concentrations—from 1.8 to 86 mg/m^3. It is surprising that exposures to freshly generated amorphous silica nanoparticles at concentrations of 86 mg/m^3 for 3 days did not produce pulmonary inflammatory responses in the lungs of exposed rats. The results presented herein demonstrate a technique for generating aerosols of *de novo* synthesized nanoparticles at two different size ranges and extrapolating to mass concentration metrics. This process of engineered nanoparticle synthesis and exposure development can be utilized for inhalation hazard studies and obviates the issue associated with generating agglomerated particle-types. However, this technique may not be fully suitable for quantifying exposures to powder aerosols, and may require additional, complementary techniques, such as cascade impactor–based analyses and/or gravimetric endpoints. Nonetheless, although the present study was a short-term effort, the methodology described herein can be utilized for longer-term inhalation toxicity studies in rats such as 28-day or 90-day studies. The expansion of the concept to subchronic studies is practical, due, in part, to the consistency of the nanoparticle generation method.

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Declaration of interest

This study was supported by the DuPont Company. The authors alone are responsible for the content and writing of the paper. The authors report no declarations of interests.

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