Video Article

Whole-Body Nanoparticle Aerosol Inhalation Exposures

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Abstract

Inhalation is the most likely exposure route for individuals working with aerosolizable engineered nano-materials (ENM). To properly perform nanoparticle inhalation toxicology studies, the aerosols in a chamber housing the experimental animals must have: 1) a steady concentration maintained at a desired level for the entire exposure period; 2) a homogenous composition free of contaminants; and 3) a stable size distribution with a geometric mean diameter < 200 nm and a geometric standard deviation $\sigma_g < 2.5$ [5]. The generation of aerosols containing nanoparticles is quite challenging because nanoparticles easily agglomerate. This is largely due to very strong inter-particle forces and the formation of large fractal structures in tens or hundreds of microns in size [6], which are difficult to be broken up. Several common aerosol generators, including nebulizers, fluidized beds, Venturi aspirators and the Wright dust feed, were tested; however, none were able to produce nanoparticle aerosols which satisfy all criteria [5].

A whole-body nanoparticle aerosol inhalation exposure system was fabricated, validated and utilized for nano-TiO_2 inhalation toxicology studies. Critical components: 1) novel nano-TiO_2 aerosol generator; 2) 0.5 m^3 whole-body inhalation exposure chamber; and 3) monitor and control system. Nano-TiO_2 aerosols generated from bulk dry nano-TiO_2 powders (primary diameter of 21 nm, bulk density of 3.8 g/cm^3) were delivered into the exposure chamber at a flow rate of 90 LPM (10.8 air changes/hr). Particle size distribution and mass concentration profiles were measured continuously with a scanning mobility particle sizer (SMPS), and an electric low pressure impactor (ELPI). The aerosol mass concentration (*C*) was verified gravimetrically (mg/m^3). The mass (*M*) of the collected particles was determined as $M = (M_{post}-M_{pre})$, *where* M_{pre} and M_{post} are masses of the filter before and after sampling (*mg*). The mass concentration was calculated as $C = M/(Q*t)$, where *Q* is sampling flowrate (m^3/min), and *t* is the sampling time (*minute*). The chamber pressure, temperature, relative humidity (RH), O_2 and CO_2 concentrations were monitored and controlled continuously. Nano-TiO_2 aerosols collected on Nuclepore filters were analyzed with a scanning electron microscope (SEM) and energy dispersive X-ray (EDX) analysis.

In summary, we report that the nano-particle aerosols generated and delivered to our exposure chamber have: 1) steady mass concentration; 2) homogenous composition free of contaminants; 3) stable particle size distributions with a count-median aerodynamic diameter of 157 nm during aerosol generation. This system reliably and repeatedly creates test atmospheres that simulate occupational, environmental or domestic ENM aerosol exposures.

Video Link

The video component of this article can be found at https://www.jove.com/video/50263/

Protocol

The whole-body nanoparticle inhalation exposure step-by-step operating procedures are described as follows.

Note: 1) steps 1 and 3 should be performed in a fume hood; 2) operators must wear appropriate personal protective equipment (respirators, goggles and rubber gloves).

1. Conditioning TiO_2 Nanoparticle Dry Powders

1. Place nano-TiO_2 powders in a nontransparent container.
2. Leave the container lid open.
3. Place the container in a dry desiccator for at least 24 hr for conditioning.

2. Warming up Data Acquisition and Control System, SMPS and ELPI and All Transducers

1. Turn on the air monitoring and data acquisition system and power switches for aerosol monitoring SMPS (TSI Inc., Shoreview, MN) and ELPI (Dekati, Tampere, Finland), and warm the systems up for at least 1 hr.
2. Turn on the power switches in all transducers to warm them up for at least 1 hr.

3. Loading TiO_2 Nanoparticle Dry Powders into Aerosol Generators

1. Open the cylinder caps on the aerosol generators, and replace the filters in the aerosol generators. Note: One aerosol generator has one cylinder. The number of aerosol generators to be used depends on the desired mass concentration of the particles in exposure chamber.
2. Weigh ~4 g nano-TiO_2 powders and load them in each cylinder.
3. Replace the cylinder caps.
4. All areas suspect of TiO_2 contamination should be wet wiped.

4. Connecting Aerosol Generators to Inhalation Exposure Chamber

1. Connect all the outlets of the aerosol generators via a manifold to a cyclone separator which is at the inlet of the inhalation exposure chamber (TSE Systems GmbH, Bad Homburg, Germany).
2. Connect compressed air tubing to the Venturi dispersers in the aerosol generators.

5. Connecting Air Monitoring and Aerosol Sampling Inlets to the Inhalation Exposure Chamber

1. Connect temperature & relative humidity (RH), pressure, O_2 & CO_2 sensors supplied by TSE Systems to test atmosphere monitoring ports on the inhalation exposure chamber.
2. Connect the inlet of an aerosol dilutor to one of the aerosol sampling ports on the inhalation exposure chamber, and then connect its outlet to the inlet of the ELPI.
3. Connect SMPS to one of the aerosol sampling ports on the inhalation exposure chamber.
4. Connect inlet of a particle concentration monitor (TSE Systems) to one of the aerosol sampling ports on the exposure chamber.
5. Weigh PTFE membrane filter (P/N 66149, Pall corporation, Ann Arbor, Michigan) and load filter into a stainless steel filter holder (In-Tox products, Moriarty NM).
6. Connect the inlet of the stainless steel filter holder with a pre-weighed filter to one of the aerosol sampling ports on the inhalation exposure chamber, and connect its outlet to a sampling pump.

6. Activate Data Acquisition Systems

1. Activate ELPI data acquisition software, *ELPIVI*, check setup parameters, and turn on the flush pump for ~5 min and then zero the ELPI. Record pre-exposure concentration.
2. Activate SMPS data acquisition software. Record pre-exposure concentration.
3. Activate software, *Daco* (TSE Systems), for monitoring and controlling air flow rate, temperature and RH chamber pressure, temperature & RH, O_2 and CO_2.

7. Loading Experimental Animals into the Inhalation Exposure Chamber

1. Weigh the experimental animals.
2. Mark the experimental animals and cages so that the animals can be put back in the same cages after the exposure if needed.
3. Open the door of the inhalation exposure chamber, and load experimental animals into the wired cages.
4. Water may be provided for animals.
5. Close and secure the door of the inhalation exposure chamber.
6. Frequently observe animals through the exposure chamber observation windows for signs of distress. Animals should be relaxed and behaving normally. Stop the exposure if rapid/labored breathing, abnormal appearance, postural abnormalities or immobility are observed. Remove the animals, return them to their original cages, contact attending veterinarian and/or initiate appropriate Institutional Animal Care and Use Committee procedures.

Note: Operators must wear personal protective equipment when performing steps 8.7, 8.8 and 8.17.

8. Exposing Small Animals to Nanoparticle Aerosols

1. Turn on the exhaust vacuum pump of the inhalation exposure chamber.
2. Run data acquisition software, *Daco*, to: a) supply filtered dry air to the exposure chamber, b) control the pressure in the exposure chamber, and c) collect the data of the exposure environment, such as pressure, temperature, RH, O_2 and CO_2.
3. Establish a slightly negative pressure (set point = -0.2 mbar) in the chamber pressure.
4. Turn on the aerosol generators.
5. Run ELPI and SMPS data acquisition software to continuously monitor particle size and relative mass concentration in the inhalation exposure chamber.

6. When the aerosol concentration is stable, *i.e.* the concentration profile on ELPI monitor reached plateau (Normally: this takes 20 min after the aerosol generators are in operation), set up the sampling time (for example, 1 hr) and turn on the aerosol sampling pump to collect representative sample of nanoparticles with filters.
7. Once the sampling time is reached, remove the filters and plug the sampling ports with rubber plugs to prevent test materials from escaping the exposure chamber.
8. Weigh the filters, and calculate the mean mass concentration in the exposure chamber as described above.
9. If the mean concentration is off the targeted concentration, manually adjust the airflow in the generators to ensure the targeted concentration is attained.
10. Calculate particle deposition in the animal lungs as $D = C \times V_m \times t \times F_r$, where D = Dose, C = mean mass concentration of test material, V_m= minute volume, t = exposure duration, and F_r = fraction of material that is deposited or absorbed.
11. Replace the filters in the filter holders with clean, pre-weighted filters, and repeat steps 8.6 and 8.8.
12. Based upon the real mass concentration in the exposure chamber and targeted particle deposition in the animal lungs, estimate the remaining exposure time as, $t_{remain} = (D_{targeted} - D) / (C \times V_m \times F_r)$, where t_{remain} = remain exposure duration, $D_{targeted}$ = targeted dose, C = mean mass concentration of test material, V_m = minute volume, F_r = fraction of material that is deposited or absorbed.
13. Turn off the aerosol generator when t_{remain} is reached.
14. Before removing the animals from the exposure chamber, flush the inhalation exposure chamber with the filtered air until the particle concentration indicated in the monitor is close to the pre-exposure particle concentration in the chamber.
15. Turn off the chamber exhaust vacuum pump.
16. Stop data acquisition software, *Daco*.
17. After exposure, observe animals to verify normal respiration and behavior, and document that no other study complications exist. If nasal discharge, respiratory distress or any other animal welfare complications are observed, contact attending veterinarian and/or initiate appropriate Institutional Animal Care and Use Committee procedures.
18. Stop ELPI and SMPS data acquisition software.

9. Creating Test Report

9.1 Test conditions include

1. Description of the aerosol generation system and its operating parameters used in this test.
2. Description of the exposure apparatus including design, type, dimensions and its operating parameters used during the exposure.
3. Equipment for measuring temperature, humidity, particle size, and actual concentration.
4. Treatment of exhaust air and the method of housing the animals in the test chamber when used.

9.2 Exposure atmosphere data include

1. Airflow rates through the inhalation equipment.
2. Temperature and humidity of the air.
3. Actual (analytical or gravimetric) concentration in the aerosol sampling zone which is near the animal cages.
4. Particle size distribution, and calculated count median aerodynamic diameter and geometric standard deviation.
5. Explanation as to why the desired chamber concentration and/or particle size could not be achieved (if applicable), and the efforts taken to comply with these aspects of the guidelines.

9.3 Other

1. Slightly negative pressure in the room containing inhalation facility should be maintained to prevent test materials from escaping inhalation exposure lab.
2. Clean the exposure chamber daily to eliminate the influences of the animal wastes.
3. ELPI, SMPS and other instruments should be cleaned and calibrated based on the user manuals.

Representative Results

An inhalation exposure study typically involves maintaining an experimental animal in a known and constant test environment while exposing the experimental animal to a defined concentration of a test material [8,9]. The whole-body nanoparticle inhalation exposure system is shown in **Figure 1**. The whole-body chamber was operated on a dynamic flow basis where there was a 90 LPM continuous flow of air through the chamber. This air flow provided 10.8 air changes/hr which exceeds the minimum number of air exchanges (10.0) required by U.S. *Environmental Protection Agency* for acute inhalation exposures [7]. A 3-stage air filter system, including a coalescing filter, a high efficiency coalescing filter and an active carbon filter (Atlas Copco, Sweden), was used on inlet air for removal of water, dust and oil vapors and (hydrocarbon) odors. A 3-stage air filter system including a pre-paper filter, a charcoal filter and a HEPA filter was used to protect exhaust mass flow controller. Per West Virginia University's request, a 4-stage air filter system designed by TSE Systems was used at outlet of the exhaust vacuum pump. The exposure chamber has a capacity of housing 8 animal cages that were made of stainless steel wire and supplied by TSE Systems. The maximum number of experimental animals immersed in the atmosphere in the exposure chamber is 16 rats, or 64 mice. The total volume of the experimental animals does not exceed 5% of the volume of the chamber to ensure stability of a test atmosphere, which is required by U.S. *Environmental Protection Agency* for acute inhalation exposures [7].

A nanoparticle aerosol generator was designed and tested [3,10]. It consists of a vibrating fluidized bed cylinder (5) with a baffle (4), a vibrating Venturi disperser (6) and a cyclone separator, as shown in **Figure 2**. A vibrator (10) attached to the cylinder (5) produces mechanical vibrations. A filter (2) sits on the stainless steel air distributor (1) in the cylinder. Nanoparticle dry powder (3) to be aerosolized rests on the filter. The Venturi disperser (6) connected to the exit port on the top of the cylinder. The Venturi disperser has a constriction in a pipe. A high-velocity air jet blowing across the constriction in the Venturi disperser can create a vacuum in the cylinder, which draw the clean and dry air into the cylinder from the air

feed ports on the both proximal and distal ends through an activated carbon and HEPA filter (9). The Venturi disperser outlet is connected to the inlet of a cyclone separator (7). The outlet of the cyclone separator is connected to the inlet of the exposure chamber. In this aerosol generation system, vibrating shear flows and multiple impactions are utilized to disperse larger agglomerates, multiple particle separators used to remove the large agglomerates, and multiple dilutions used to minimize re-agglomeration of the particles. The particle size and mass concentration can be controlled by manually adjusting the vibrations and air flow rates through dry powder layer via valves (8) and (11).

TiO_2 aerosols generated from nano-TiO_2 bulk dry powder (Aeroxide TiO_2 P25, Evonik, Germany) were diluted and delivered to the inhalation exposure chamber at 90 LPM. The test atmospheres were monitored with the ELPI and adjusted manually to assure a consistent and known exposure for each experimental animal group. In addition, a sham group consisting of the same number of experimental animals should always be included in the study. The control experimental animals will be exposed to clean filtered air instead of aerosol particles and the results from this sham group would be used to evaluate the biological effects of the test nanoparticle aerosol on the experimental animals.

1. Chamber Pressure

The chamber pressure was monitored with a pressure transducer. Slightly negative pressure (-0.2 ± 0.01 mbar) inside the chamber, as shown in **Figure 3,** was maintained through controlling the chamber inlet and outlet air flow rates to prevent leakage of the test substance into the surrounding laboratory. Ideally the room containing the inhalation exposure chamber should be at a slightly negative pressure.

2. The Air Flow Rates, Temperature and Relative Humidity

The inlet and exhaust air flow rates were controlled by mass flow controllers. As shown in **Figure 4,** the inlet air flow rate was 89.9 ± 0.3 LPM, and exhaust air flow rate was 111.9 ± 0.9 LPM. The temperature and relative humidity were monitored with a temperature and RH transducer and controlled at 22.6 ± 0.4 °C and 6.9 ± 0.6% through controlling the room air temperature and with a humidifier, as shown in **Figure 5**. According to Pauluhn & Mohr's investigations under the relative humidity between 3 and 80%, rats tolerated either humidity atmosphere without any specific effects [4].

3. Chamber O_2 and CO_2 Concentrations

The O_2 and CO_2 concentrations were monitored continuously with a O_2 and a CO_2 gas analyzers. As shown in **Figure 6**, O_2 was stable at 20.79 ± 0.03%, and CO_2 concentration was 580 ± 25 ppm.

4. Aerosol Characterization

An aerosol used for inhalation studies is commonly characterized in real time by two parameters that describe the size distribution function and a concentration parameter. A continuous flow of the test atmosphere was pulled from the zones just above the animal cages in the chamber through a sample line to the analysis instrument.

4.1 Particle Size Distribution

Figure 7A is the particle size distribution measured with a standard 10 LPM ELPI. The count median aerodynamic diameter of the particles is 157 nm. **Figure 7B** is the particle size distribution measured with TSI 3936L75 SMPS. The count median mobility diameter of the particles is 145 nm with a geometric standard deviation of 2.3. **Figure 7C** shows the particle size change during the inhalation exposure studies. The particle size is relatively stable during the whole exposure period.

4.2 Aerosol Concentration

The real-time mass concentration profile of the nano-TiO_2 particles was monitored in the zones just above the cages with an ELPI. **Figure 8A** is the particle concentration during a 4 hr/day inhalation exposure. During the inhalation exposure, the actual concentrations were measured using gravimetric methods, three to four measurements were taken, for calculating inhaled dose. The particles were collected with 47 mm PTFE membrane filters. A XP2U microbalance (Mettler Toledo, Switzerland) was used to weigh the fillers.

The intra-day and inter-day variability of nano-TiO_2 concentration in the inhalation exposure chamber were determined based on gravimetric concentrations of 29 individual 4 hr/day inhalation exposures (targeted concentration = 6.0 mg/m^3). Each intra-day mean concentration and its relative standard deviation (RSD) were calculated based on 3 or 4 gravimetric measurements during that 4 hr inhalation exposure, as shown in **Figure 8B**. The intra-day concentration has a mean of 5.3 to 6.6 mg/m^3 with RSD between 0.02 and 0.17. The mean inter-day concentration and its RSD were calculated based on 29 individual mean intra-day gravimetric concentrations. The inter-day mean concentration is 6.0 mg/m^3 with a RSD of 0.06. It indicated that our system can provide stable and reproducible nano-TiO_2 test atmospheres for acute inhalation exposures.

4.3 Aerosol Morphology and Elemental Composition

Structures and chemical compositions of particles are critical in toxicology studies. TiO_2 samples were collected onto 47-mm Nuclepore polycarbonate filters (Whatman, Clinton, PA). The filters were cut into four equal sections; two sections were mounted onto aluminum stubs with silver paste (Colloidal silver liquid, Electron Microscopy Sciences, Hatfield, PA). The deposited TiO_2 particles were viewed using a Hitachi 4800 field emission scanning electron microscope (FESEM, Hitachi, Japan), and also analyzed using energy dispersive X-ray analysis (SEM-EDX; Princeton Gamma-Tech, Rocky Hill, N.J.) at 20 keV. **Figure 9** is SEM micrographs of TiO_2 aerosol samples, and **Figure 10** is a spectrum for the TiO_2 aerosol samples. More than a hundred particles were examined with the SEM-EDX to ensure that particles on the filter were truly composed of titanium and oxygen, an indication of TiO_2 particles. In **Figure 10**, the carbon is from the filter and the gold/palladium is from the coating. Based on the SEM-EDX results, all the particles examined consisted of titanium and oxygen only, demonstrating that they were truly TiO_2 particles.

5. Uniformity of Distribution

Maintaining the appropriate environmental parameters inside the chamber is insufficient if the concentration of test compound varies from location to location [3]. The nanoparticle concentrations were measured at four different locations in the zones just above the cages in the exposure chamber.

Mass of the particles at a location, M_i, was measured gravimetrically with filter sampling and a micro-balance. The mean mass of the sampled particles is

$$M_{mean} = \frac{1}{N}\sum_{i=1}^{N} M_i$$

The relative deviation of the mass concentration at location *i* from the mean concentration is

$$E_i = \frac{|M_i - M_{mean}|}{M_{mean}} \times 100\%$$

The maximum relative deviation of the concentrations at different measurement locations from the mean concentration is < 6%. This is within tolerance limits for group calculation.

6. Calculated Particle Deposition in Animal Lungs

If the animal is inhaling a known concentration of test atmosphere during the exposure period and the uptake or deposited fraction is known, the amount of deposited test material can be calculated:

$$D = C \times V_m \times t \times F_r$$

where D = Dose, C = concentration of test material, V_m = minute volume, t = exposure duration, and F_r = fraction of material that is deposited or absorbed.

Average values for the minute volume, V_m can be estimated from body mass using empirical allometric scaling formulae [1,2]. For example, assuming a rat has a minute ventilation V_m = 200 ml/min, exposure concentration C = 6.2 mg/m^3, exposure duration t = 4 hr, fraction of the material deposition F_r = 0.1, then the calculated lung deposition D = 30 µg.

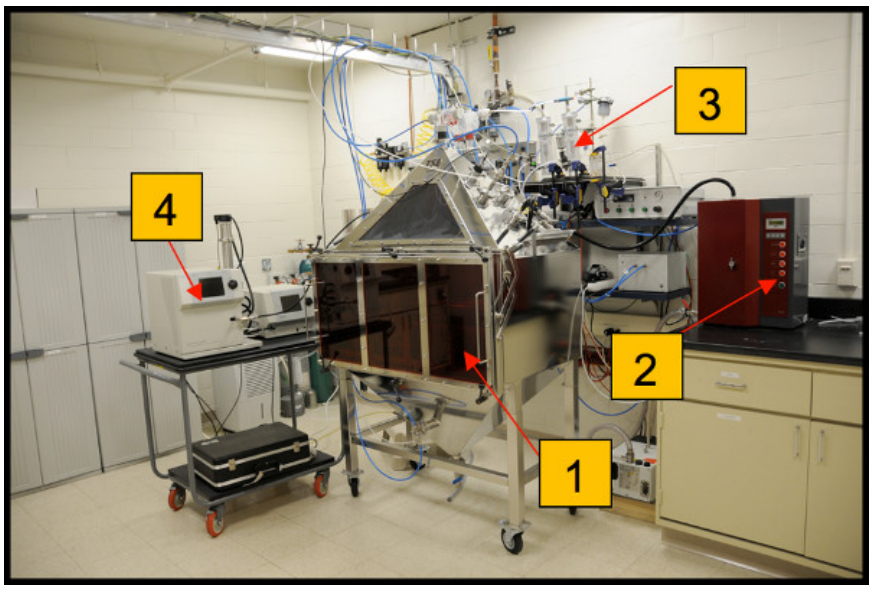

Figure 1. Inhalation Exposure Facility. 1 = Exposure chamber; 2 = Electric low pressure impactor; 3 = Aerosol generator; 4 = Scanning mobility particle sizer.

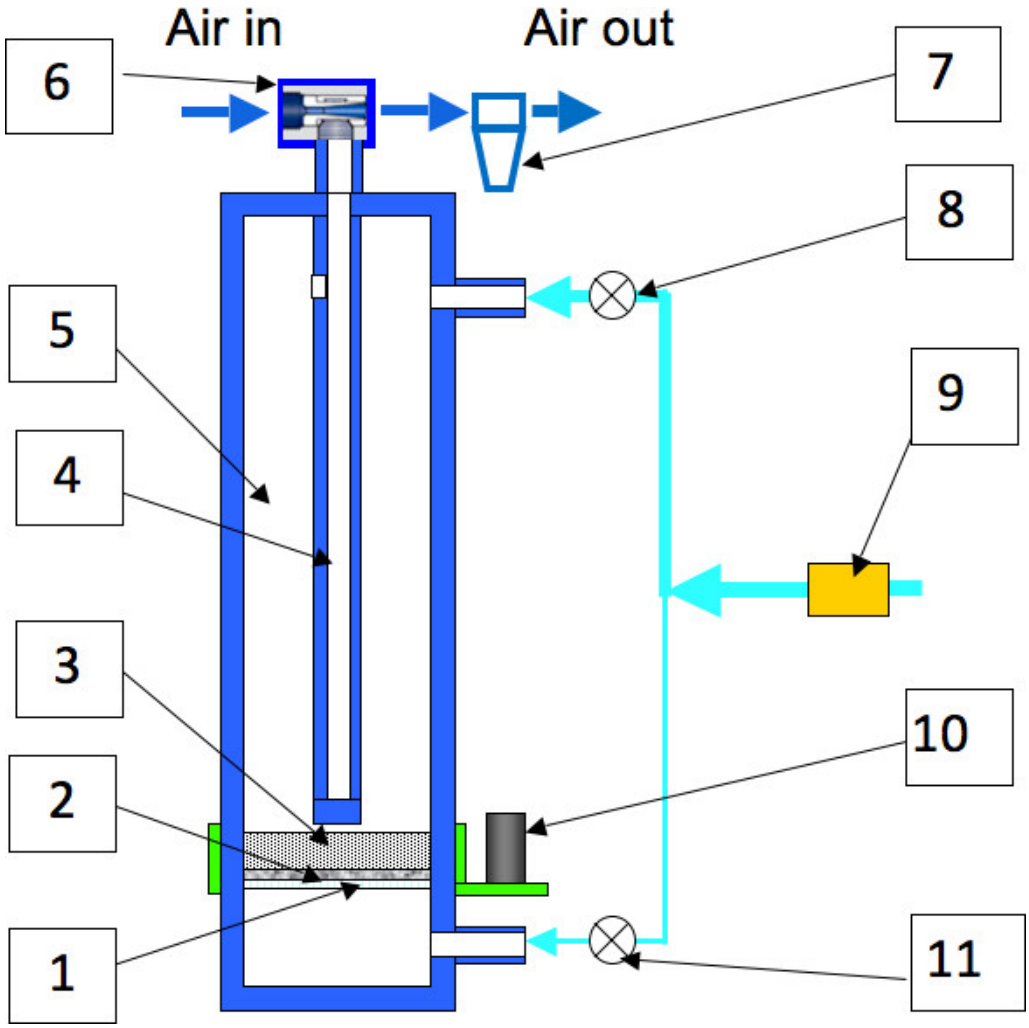

Figure 2. Schematic diagram of nano-TiO2 aerosol generator. 1 = air distributor; 2 = filter; 3 = TiO_2 dry powder; 4 = baffle; 5 = cylinder; 6 = Venturi disperser; 7 = cyclone separator; 8 = valve (dilution air); 9 = charcoal & HEPA filter; 10 = vibrator; 11 = valve (air through dry powder).

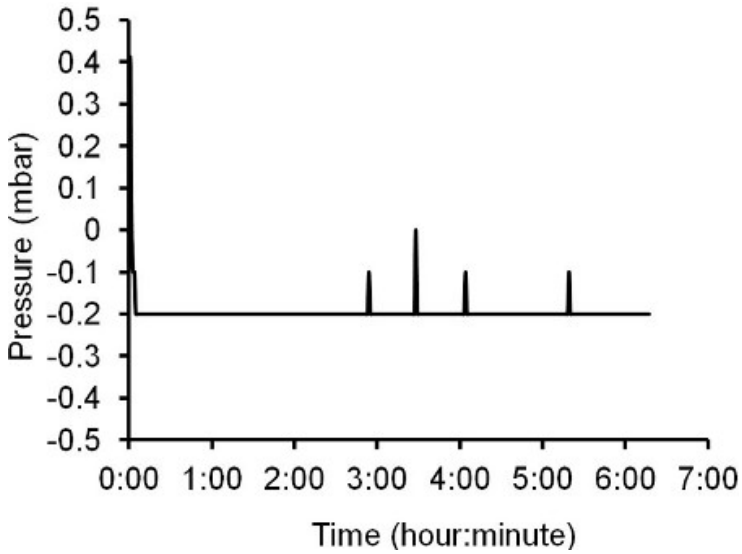

Figure 3. Chamber pressure. A slightly negative pressure in the chamber was maintained at -0.2 mbar (targeted pressure). Once the pressure is off the targeted pressure (spikes), the control system adjusted the pressure back to the targeted pressure.

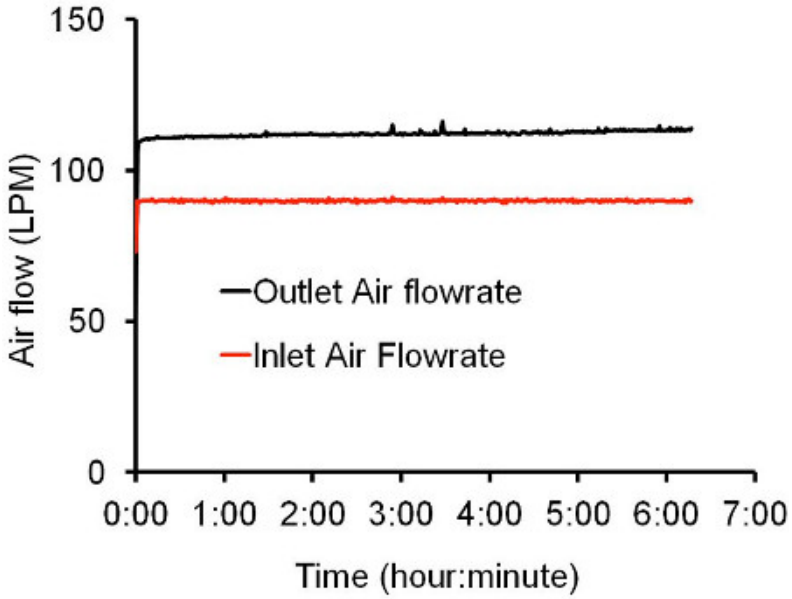

Figure 4. Chamber inlet and outlet air flow rates. Mean inlet air flow rate = 89.9 LPM, and exhaust air flow rate = 111.9 LPM to maintain a slightly negative pressure in the chamber.

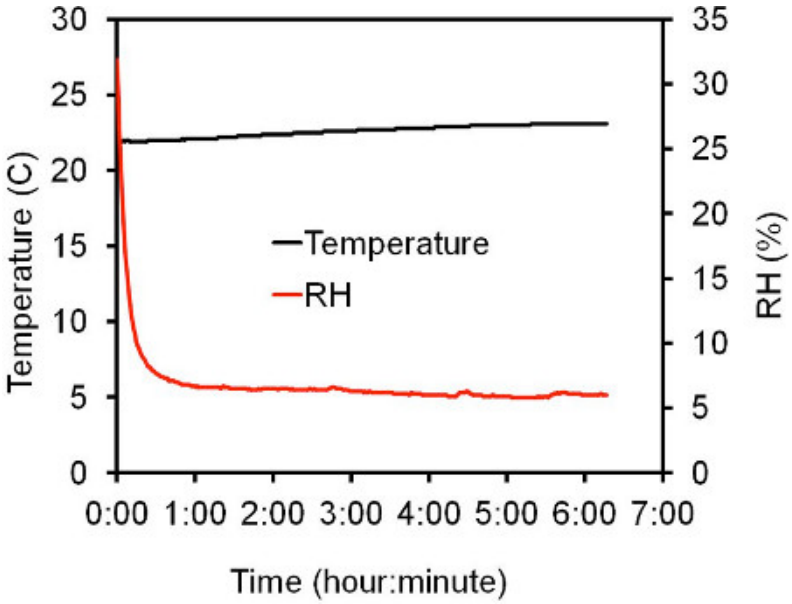

Figure 5. Chamber temperature and RH. The mean temperature = 22.6 ± 0.4 °C, while the RH is 6.9 ± 0.6%.

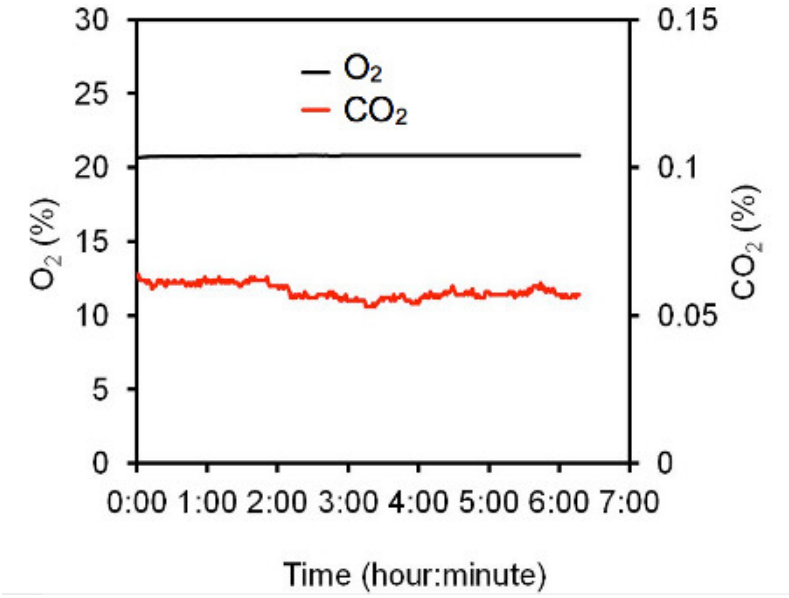

Figure 6. Chamber O_2 and CO_2. The O_2 is 20.79%, and CO_2 is 580 ppm.

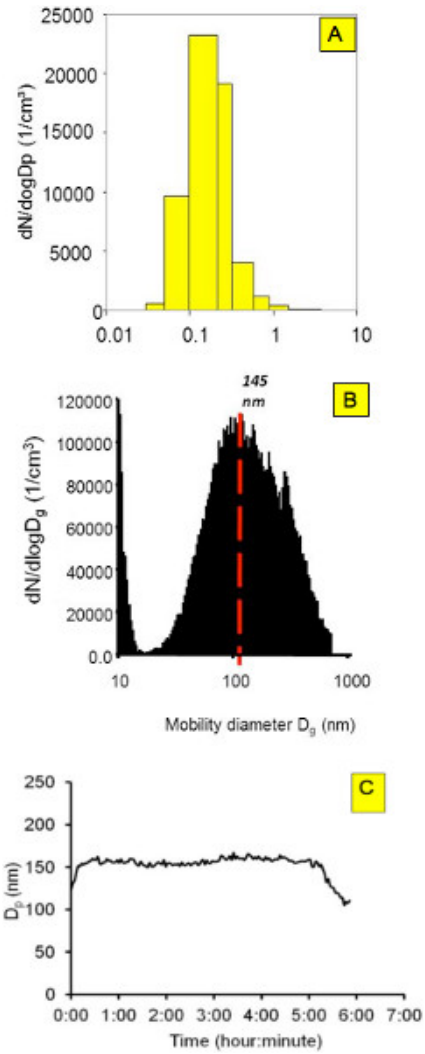

Figure 7. TiO_2 aerosol size distribution. A) ELPI, count median aerodynamic diameter D_p = 157 nm; **B)** SMPS, count median mobility diameter D_g = 145 nm with a geometric standard deviation σ_g of 2.3. **C)** Particle size vs. time from ELPI. Click here to view larger figure.

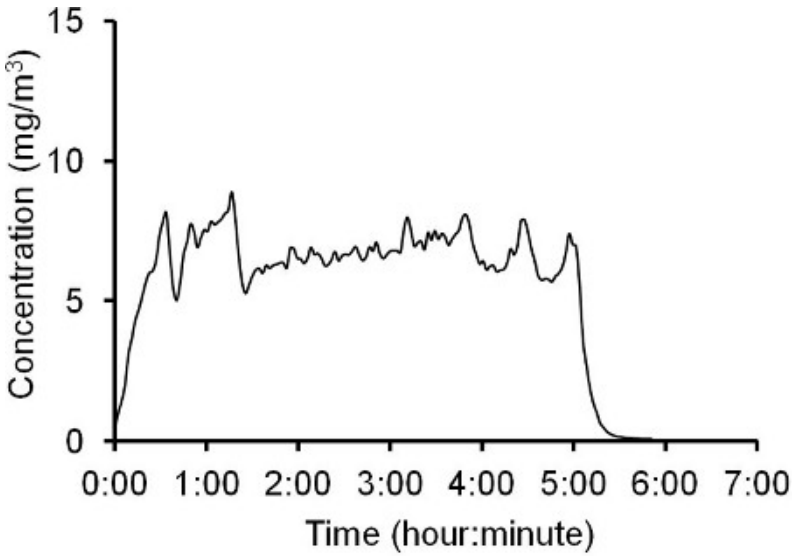

Figure 8A. 4 hr TiO_2 aerosol mass concentration.

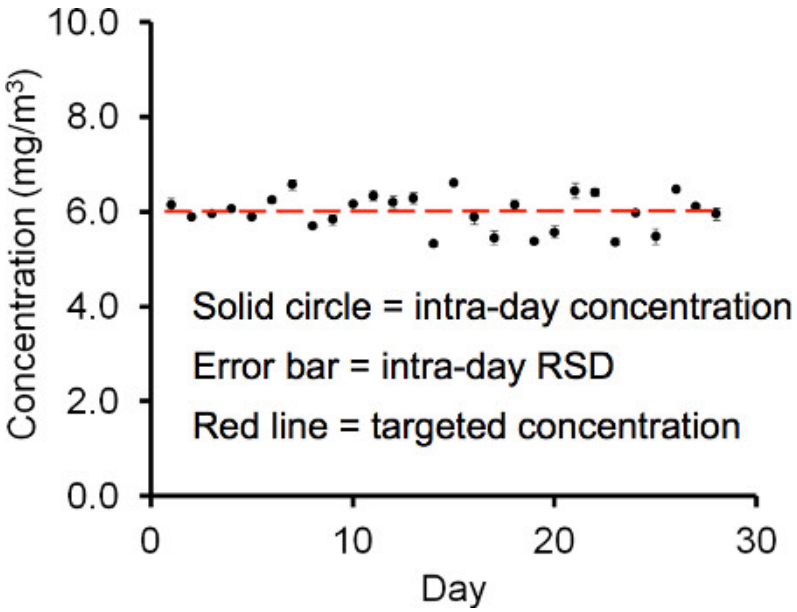

Figure 8B. TiO_2 aerosol mass concentrations of 29-individual 4 hr inhalation exposure.

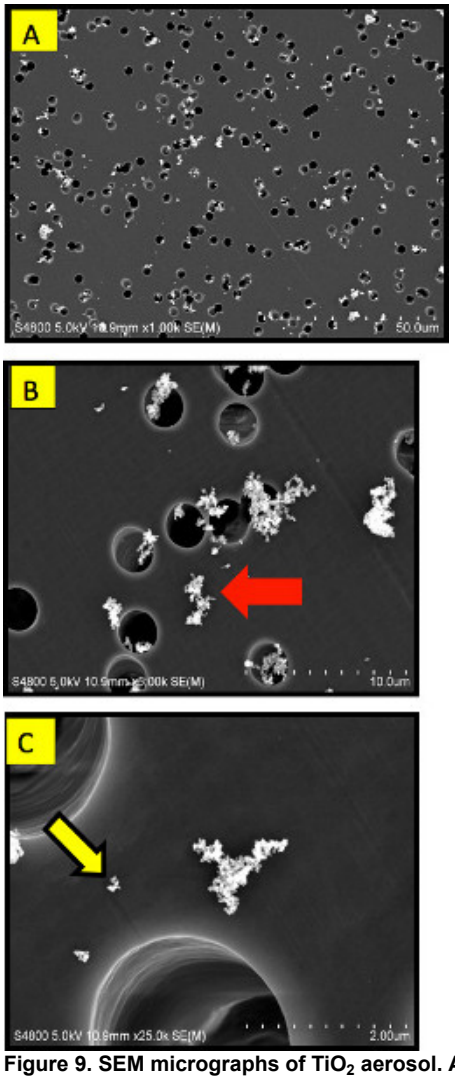

Figure 9. SEM micrographs of TiO_2 aerosol. A) Typical particle distribution on 47 mm filter. **B)** Red arrow, 1.78 µm. **C)** Yellow arrow, 159 nm. Click here to view larger figure.

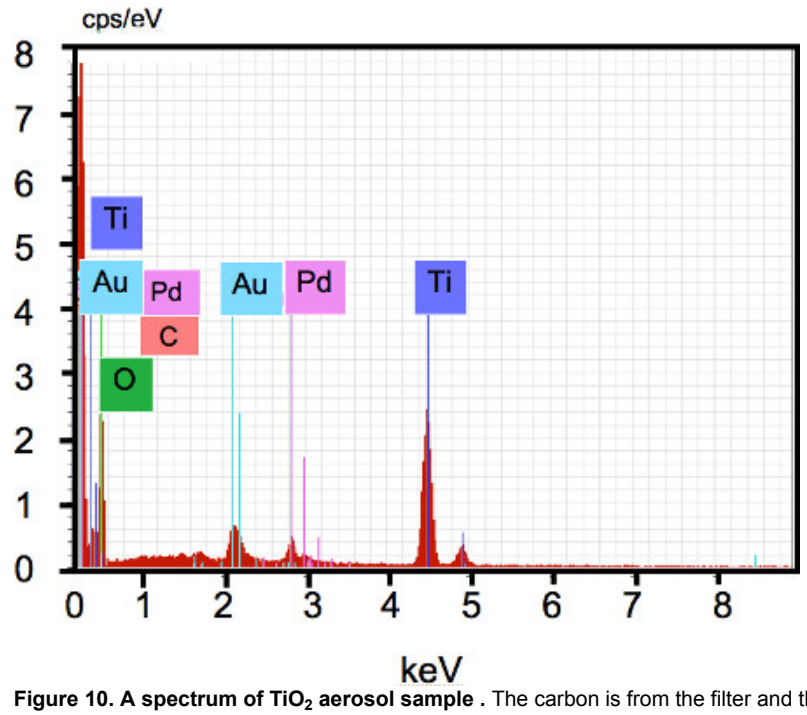

Figure 10. A spectrum of TiO_2 aerosol sample . The carbon is from the filter and the gold/palladium is from the coating. Based on the SEM-EDX results, all the particles examined consisted of titanium and oxygen only, demonstrating that they were truly TiO_2 particles.

Discussion

We have assembled and described here in a whole-body nanoparticle aerosol inhalation exposure system. The system functionality was validated with state-of-the-art nanoparticle aerosol characterization techniques. With a novel nanoparticle aerosol generation system, this inhalation exposure system can provide a well characterized, controlled and uniform nanoparticle aerosol test atmosphere with relatively consistent temperature, humidity, air flow, and oxygen content for experimental animals. The exposure system is most efficient for large numbers of animals, or long term studies. In this large whole-body chamber, experimental animals are unrestrained, comfortable and heat stress is minimized. The major limitation of the exposure is that the experimental animals are immersed in the atmosphere in the exposure chamber. Other routes of exposure such as oral and dermal exposure can occur. Also, in the whole-body system, large amount of bulk material is required because of larger inlet flow rate. For instance, in this system with a 0.5 m^3 exposure chamber, the inlet air flow rate is 90 LPM, while for a 12-port nose-only inhalation exposure system, the inlet air flow rate is 12 LPM. Therefore, cost and availability of bulk materials must be considered when planning inhalation exposure studies.

Disclosures

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health. The mention of any company names or products does not imply an endorsement by NIOSH, nor does it imply that alternative products are unavailable, or unable to be substituted after appropriate evaluation.

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