Thermal Spray Coating Aerosol Generation and Exposure System

-An automated, computer-controlled thermal spray coating particle generator and inhalation system was designed and constructed to perform animal studies to mimic workplace exposures.

-The mass concentration in the chamber was monitored by a real time aerosol monitor (DataRAM) and kept below 5000 ppm. The sensors and measurement devices were managed and controlled through a custom computer software programed written in LabVIEW.

-Ports installed inside the animal chamber were used for gravimetric sampling pumps to also determine particle concentration using cassettes with 47-mm filters. This filter data was used every exposure run to validate and calibrate the DataRAM. The target exposure chamber mass concentration could be selected in the software and was typically set to 25 to 30 mg/m3 for all experiments.

-The physicochemical properties of aerosols generated during electric arc wire- thermal spray coating using commonly used consumable wire PMET 720. In addition, a study of PMET 720 using two different compressed air pressures (50 and 60 psi) was performed to assess whether it affected the physical and/or chemical characteristics of the generated particles.

Thermal Spray Coating Aerosol Characterization

-Particle size and morphology. The size distribution of the different thermal spray coating aerosols inside the exposure chamber were determined by Micro-Orifice Uniform Deposit Impactor (MOUDI) To assess particle morphology, the different thermal spray coating particles were collected onto 47-mm filters and were viewed using a Hitachi S4800 field emission scanning electron. The MOUDI impactor has size cutoff filters 0.056, 0.1, 0.18, 0.32, 0.56, 1, 1.8, 3.2, 5.6, 10 and 18 μm. Elemental profiles were determined by energy dispersive X-ray spectroscopy analysis to map specific metal components of the particle samples.

-Metal Composition. Particle samples were collected inside the exposure chamber onto filters in 37-mm cassettes during thermal spray coating using the different consumable wires. The particle samples were digested, and the metals analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

In vitro Cytotoxicity, membrane damage, uptake and screening for various mechanisms of toxicity

- RAW 264.7 Monocyte/Macrophage cell line was obtained from American Type Culture Collection (Manassas, VA; ATCC TIB-71). The cells were cultured in Dulbecco's Modified Eagle medium (DMEM, Invitrogen, Waltham, MA) supplemented with 10% Fetal Bovine Serum (FBS, R & D Systems, Minneapolis, MN) and 1% Penicillin-Streptomycin (Invitrogen, Waltham, MA) Subcultures were prepared by scraping and a sub cultivation ratio of 1:4 was maintained. Cell culture media was replaced every 2-3 days.

-Cytotoxicity and membrane damage was evaluated by exposing the cells to 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 μg/ml of the particulate for 24 hrs.

-Cytotoxicity due to particle exposure was accessed in RAW 264.7 cells by measuring the cell's ability to reduce tetrazolium salt WST-1. The cells were exposed to particulates for 24 hrs and challenged with fresh media containing 10% volume/volume WST-1 cell proliferation reagent (Sigma-Aldrich, St. Louis).

- Membrane integrity of the cells after particulate exposure was assessed using CytoTox-One homogenous membrane integrity assay (Promega, Madison, WI).

-Intracellular oxidative stress due to particle exposure was determined using CellROX® Green (Invitrogen, Waltham, MA). Cells were exposed to 0, 6.25, 25 and 100 μg/ml of the particulate for 24 hrs.

- The mechanism of uptake of the welding fume spray particulate was evaluated in RAW 264.7 cells by challenging them with 15 μM chlorpromazine (Invitrogen, Waltham, MA), 3 μM filipin III (Cayman Chemical Company, Ann Arbor, MI) or 8 μM cytochalasin B (Sigma-Aldrich, St. Louis, MO). These pharmaceuticals inhibited clathrin-mediated endocytosis, caveolin-mediated endocytosis and actin-dependent pinocytosis/phagocytosis respectively. Cells were preincubated for 30 minutes with fresh media containing the inhibitors or media alone and then co-challenged with 50 μg/ml of particulate with and without the inhibitors for 3 hr. The cells were lifted and washed, and uptake was quantified by determining the granularity by flow cytometry/

- Monocyte/macrophage reporter cell line, RAW-Blue™ Cells (Invivogen, San Diego, CA) were used to evaluate NF-κB and AP-1 induced due to particulate exposure. Cells were exposed to 0, 1.56, 3.125, 6.25 and 12.5 μg/ml for 16 hours.

<u>In vivo Exposure, Assessment of Lung Toxicity and Inflammation</u>

-Male Sprague-Dawley rats (250 - 300 g) were used and free of viral pathogens, parasites, mycoplasmas, Helicobacter, and CAR Bacillus. The rats were acclimated for one week after arrival and provided food and water ad libitum.

-The rats were exposed to aerosols (25 mg/m3 x 4 hr/d x 9 d) generated from electric arc wire- thermal spray coating using a stainless-steel consumable wire (PMET720) at settings of 200 A, 60 psi, and 30 V. Control animals were exposed to filtered air. At 1, 7, 14 and 28 d after the final exposure, bronchoalveolar lavage (BAL) was performed to assess lung injury and inflammation.

-Animals were euthanized with an overdose of a pentobarbital-based euthanasia solution (>100 mg/kg body weight, IP; Fatal-Plus Solution, Vortech Pharmaceutical, Inc., Dearborn, MI, USA) and then exsanguinated by severing the abdominal aorta.

-The right lung was first lavaged with a 1 ml/100 g body weight aliquot of calcium- and magnesium-free phosphate-buffered saline (PBS), pH 7.4. The first fraction of recovered bronchoalveolar lavage fluid (BALF) was centrifuged at 500 x g for 10 minutes, and the resultant cell-free supernatant was saved for lung cell damage analysis. The right lung was further lavaged with 6 ml aliquots of PBS until 30 ml were collected. These samples also were centrifuged for 10 minutes at 500 x g and the cell-free BALF discarded. The cell pellets from all washes for each rat were combined, washed, re-suspended in 1 ml of PBS buffer, counted, and differentiated.

-For the determination of lung inflammation, total cell numbers recovered by BAL were determined using a Coulter Multisizer II and AccuComp software. Cell suspensions ($5x10^4$ cells) were spun using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, England) for 5 minutes at 800 rpm onto a slide. Cells (200/rat) were identified after labeling with Leukostat stain (Fisher Scientific, Pittsburgh, PA, USA) as alveolar macrophages (AMs) and neutrophils (PMNs).

-Lactate dehydrogenase (LDH) was measured in the first fraction of the cell-free supernatant recovered from the BALF as a general marker for lung cell injury. LDH activity was determined by measuring the

oxidation of lactate to pyruvate coupled with the formation of NADH at 340 nm. Measurements were taken with a COBAS MIRA auto-analyzer.