

## **2. Materials and methods**

### **2.1. Three-dimensional printer emissions inhalation exposure system**

A custom-designed inhalation exposure system was used to deliver either HEPA-/carbon-filtered air or consumer-grade FFF 3D printer emissions to a whole-body rodent exposure chamber in real time (Farcas et al., 2020). Briefly, HEPA-/carbon-filtered air was drawn through the exposure system at 30 LPM using a vacuum and mass flow controller (MCRW-50-DS; Alicat Scientific, Tucson AZ). For control animals, HEPA-/carbon-filtered air was drawn directly into the temperature-controlled (22 – 24 °C) exposure chamber; for emissions-exposed animals, HEPA-/carbon-filtered air was drawn through an airtight chamber that housed three FFF 3D printers before arriving at the temperature-controlled exposure chamber. Emissions and conditions inside the exposure chamber were continuously monitored and controlled in real-time using external equipment that was connected to sampling ports and custom software.

### **2.2. Three-dimensional printer settings for PC filament**

The three FFF 3D printers (nozzle diameters = 0.4 mm) were programmed to simultaneously print a 12.7 x 12.7 x 2.54 cm object using black PC filament over a 4-hour period. Each print job required 240 g of filament and occupied most of the build plate surface. The printer nozzle temperatures were set to 300°C. The build plate surfaces were prepared the previous day using a thin coating of ABS juice made by dissolving 40 g of ABS filament in 100 mL of acetone, and fumes from evaporated acetone were flushed out of the chamber prior to an exposure. Filament was stored at room temperature in an airtight dry box when not in use.

### **2.3. Three-dimensional PC emissions collection and characterization**

Emissions were collected and characterized as described previously (Farcas et al., 2020). A brief overview is as follows:

For particle collection and characterization: a Data RAM (DR-1500; Thermo Electron Co., Waltham, MA) was used to continuously monitor aerosol mass concentrations inside the exposure chamber; readings were verified by gravimetric analysis (37 mm dia., 0.45 µm pore-size Teflon filters, 2 LPM). Each exposure maintained an average aerosol mass concentration of 0.529 mg/m<sup>3</sup> for 4 hours. Real-time particle counts were also recorded using a condensation particle counter (CPC; Model 3787, TSI Inc., Shoreview, MN) and associated software.

A fast mobility particle sizer (FMPS; Model 3091, TSI Inc., Shoreview, MN) was used to collect particle size data in 5-second intervals during several 4-hour mock exposures without animals in the exposure chamber. Aerosols were also collected onto track-etched polycarbonate filters (25 mm, 0.1  $\mu\text{m}$  pore-size, 1 LPM for 20 minutes) for particle morphology analysis by field emission-scanning electron microscopy (FE-SEM; Hitachi S-4800, Tokyo, Japan). In accordance with NIOSH Manual of Analytical Method (NMAM) 3900 (NIOSH 2018), emissions were sampled using fused silica-lined, evacuated canisters (450 mL; Entech Instruments Inc., Simi Valley, CA) with 3-hour capillary flow controllers, and then analyzed for specific VOCs in accordance with NIOSH Method 3900 using an Entech 7200/7650 preconcentration system coupled with an Agilent 7890/5975 gas chromatograph-mass spectrometer (GC-MS; Santa Clara, CA). Samples were collected (1/day, 5 different days) during 4-hour mock exposures without animals in the exposure chamber; average concentration and standard deviation were calculated for individual VOCs.

#### **2.4. Animals**

Male Sprague-Dawley [Hla: (SD) CVF] (SD) rats (6–7 weeks old, 200–225 g) were purchased from Hilltop Lab Animals (Scottsdale, PA), housed four per cage in ventilated polycarbonate cages, and acclimated for a minimum of seven days prior to starting the study. The facility provided a controlled environment with HEPA-filtered air,  $22 \pm 2^\circ\text{C}$  temperatures, and 40-60% humidity. The animals were fed irradiated Teklad 2918 (Harlan, Madison WI) and provided with an ALPHA-dri®/Teklad sani-chips bedding mix and tap water ad libitum. The CDC-Morgantown Institutional Animal Care and Use Committee (accredited by AAALAC International) reviewed and approved the study protocol.

#### **2.5. Experimental design**

Each of the 60 acclimated animals was assigned to one of two treatment groups—air control or 3D printer emissions exposed—for an exposure duration of 1, 4, 8, 15, or 30 days ( $n = 6$  per exposure group). For 4 h/day, 4 consecutive days/week, air control groups were subject to whole-body inhalation of HEPA-/carbon-filtered air, while 3D printer emissions-exposed groups were subject to whole-body inhalation of real-time emissions from FFF 3D printers printing with PC filament. Rats were sacrificed 24 h post-exposure via intraperitoneal pentobarbital injection (100-200 mg/kg) (Fort Dodge Animal Health; Fort Dodge, IA) followed by exsanguination via whole-blood collection from the abdominal aorta. Whole blood was immediately transferred to

two collection tubes: one EDTA-containing vacutainer for whole blood hematological analysis and one clot activator-/polymer gel-containing vacutainer for serum chemistry analysis (Becton-Dickinson; Franklin Lakes, NJ). Bronchoalveolar lavage fluid (BALF) was collected while the left lung was clamped, and the right cardiac lobe was tied off. The unlavaged right cardiac lobe and the lavaged right lobes were collected and stored at -80°C; the left lung and head/nasal tissues were preserved for histopathological evaluation.

## **2.6. Three-dimensional PC filament emission particle deposition estimates in the nasal passages, tracheobronchial and alveolar regions**

The Multiple-Path Particle Dosimetry (MPPD) model (Anjilvel and Asgharian, 1995) was used to estimate PC-emissions particle deposition mass for the head/nose, tracheobronchial, and alveolar regions, and collective deposition for tracheobronchial and alveolar regions under conditions with and without clearance.

Particle deposition mass without clearance: FMPS data was converted to mass distribution using the assumption of spherical particle shape with a density of 1.3 g/cm<sup>3</sup> (density of PC). Animal breathing rate (120 breaths/minute), tidal volume (1.7 mL), 4-hour exposure time, and 0.5 mg/m<sup>3</sup> were the parameters used for the MPPD model.

Particle deposition mass with clearance: the detailed description of these methods is provided in Farcas et al., 2020 (Farcas et al., 2020).

## **2.7. Bronchoalveolar lavage (BAL) analysis**

A brief summary of these previously described methods (Farcas et al., 2020) is as follows:

### **2.7.1. BALF fluid collection and cytology**

The rat right lung was passed with 6 mL cold PBS via a tracheal cannula; the lung was massaged for 30 seconds, the cold PBS was withdrawn, and the process was repeated a second time, yielding the first fraction of BALF. A second BALF fraction was collected by repeating the aforementioned process using 5 mL aliquots of PBS until a 15 mL volume was recovered. The two BALF fractions were centrifuged (800xg, 10 minutes, 4 °C), and the supernatant from fraction one and the cell pellets from both fractions were kept for further analysis (supernatant from fraction two was discarded). The supernatant was used to measure total protein levels, lactate dehydrogenase (LDH) activity, and surfactant and cytokine levels. The pellets were combined, resuspended in 1 mL PBS (Lonza, Pearland, TX), and used for a total cell count

(Beckman Coulter Multisizer 4 particle counter, Coulter Electronics, Hialeah, FL) and cell differential (HEMA-stained cytopsin slides, 300+ cell-count per slide).

#### **2.7.2. Transmission electron microscopy (TEM) staining of BAL cells**

BAL cells were fixed in Karnovsky's fixative, post-fixed in 2% osmium tetroxide, mordanted in 1% tannic acid, and stained en bloc in 0.5% uranyl acetate. Samples were then dehydrated with ethanol, infiltrated in propylene oxide, embedded in EPON™, and sectioned at 70 nm. Grids were stained with 4% uranyl acetate and Reynold's lead citrate and imaged using a JOEL 1400 transmission electron microscope (Tokyo, Japan).

#### **2.7.3. Scanning electron microscopy (SEM) images of lungs**

A 5 µm unlavaged lung section was mounted on a carbon planchet and deparaffinized with xylene. The sample was sputter-coated with gold/palladium and imaged using a Hitachi S4800 field emission scanning electron microscope (Tokyo, Japan).

#### **2.7.4. Total protein and LDH activity**

A Pierce™ BCA Protein Assay Kit (Fisher Scientific) and a LDH Reagent Set (Pointe Scientific, Lincoln Park, MI) were used in conjunction with a Synergy H1 Microplate Reader (BioTek, Winooski, VT) to collect total protein and LDH activity data, respectively, from the BALF samples.

#### **2.7.5. Surfactant proteins A (SP-A) and D (SP-D)**

SP-A and SP-D kits (Biomatik USA, Wilmington, DE) were used in conjunction with a Synergy H1 Microplate Reader to collect surfactant protein data from the BALF samples.

#### **2.7.6. Cytokines levels**

A V-PLEX Pro-inflammatory Panel 2 Rat Kit (MSD, Rockville, MD) was used in conjunction with a QuickPlex SQ 120 plate reader (MSD, Rockville, MD) to collect pro- and anti-inflammatory cytokine data from the BALF samples.

### **2.8. Blood processing and analysis**

Blood collected in EDTA-containing and clot-activator-/polymer gel-containing vacutainers were processed and analyzed as previously described in Farcas et al., 2020 (Farcas et al., 2020). A brief overview is as follows:

#### **2.8.1. Characterization of blood cells and hematological parameters**

A complete blood count was performed for each sample 30-45 minutes after collection using a ProCyte Dx Hematology Analyzer (IDEXX Laboratories, Inc., Westbrook, ME).

### **2.8.2. Serum chemistry profile**

Serum was extracted by allowing whole blood to clot at room temperature followed by centrifugation. Serum biochemical parameters were evaluated using a Catalyst One Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, ME).

### **2.9. Lung and nasal passages histopathological evaluation**

The unlavaged left lung was inflated with 10% neutral buffered formalin (NBF), embedded in paraffin, cut at 5  $\mu$ m and stained with Hematoxylin and Eosin (H&E) for histopathological evaluation. Lesions were reviewed by a veterinary pathologist and classified in the following manner: WNL = within normal limits; 1 = minimal change (barely exceeds WNL); 2 = mild/slight change (lesion is identifiable but is of limited severity); 3 = moderate change (lesion is prominent with a potential for increased severity); 4 = severe change (lesion occupies the majority of the organ and is as severe as possible). After processing the lungs, the nasopharynx was lightly flushed with 10% NBF, and the nasal passages were collected. Nasal tissues were fixed in formalin for 1 week, then decalcified in 13% formic acid. Standard nasal sections (T1, T2, T3, and T4) were taken (Young, 1981) and embedded in paraffin, cut at 5  $\mu$ m, and stained with H&E.