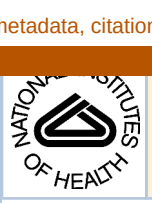

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Direct particle-to-cell deposition of coarse ambient particulate matter increases the production of inflammatory mediators from cultured human airway epithelial cells

John Volckens[1,*], **Lisa Dailey**[2], **Glenn Walters**[3], and **Robert B. Devlin**[2]

[1]Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO

[2]Clinical Research Branch, National Health and Environmental Effects Laboratory, U.S. EPA, Research Triangle Park, NC

[3]Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC

Abstract

Exposure of cultured cells to particulate matter air pollution is usually accomplished by collecting particles on a solid matrix, extracting the particles from the matrix, suspending them in liquid, and applying the suspension to cells grown on plastic and submerged in medium. The objective of this work was to develop a more physiologically and environmentally relevant model of air pollutant deposition on cultures of human primary airway epithelial cells. We hypothesize that the toxicology of inhaled particulate matter depends strongly on both the particulate dispersion state and the mode of delivery to cells. Our exposure system employs a combination of unipolar charging and electrostatic force to deposit particles directly from the air onto cells grown at an air-liquid interface in a heated, humidified exposure chamber. Normal human bronchial epithelial cells exposed to concentrated, coarse ambient particulate matter in this system expressed increased levels of inflammatory biomarkers at 1 hour following exposure and relative to controls exposed to particle-free air. More importantly, these effects are seen at particulate loadings that are 1-2 orders of magnitude lower than levels applied using traditional in vitro systems.

Introduction

Numerous epidemiological studies have demonstrated that exposure to particulate matter (PM) air pollution is associated with increased short-term cardiovascular mortality, resulting in an estimated 40,000 to 60,000 premature deaths in the United States annually. Exposure to PM is also associated with increased cardiovascular and respiratory morbidity, including exacerbation of diseases such as asthma or COPD (1). Supporting these epidemiological associations are numerous controlled human and animal exposure studies that have identified pathophysiological pathways that can explain how PM can cause adverse health effects. In vitro studies using new molecular tools and “omics” approaches are also well-suited for rapid screening of PM components, or complex PM mixtures. They are relatively inexpensive, compared with in vivo studies, and can help identify the biochemical processes that drive cellular responses to a toxicant. Consequently, in vitro models of human tissues are often used to study the effects of PM at the cellular and molecular level.

[*]Author to whom correspondence should be addressed: 1681 Campus Delivery, Fort Collins, CO 80523; ph: 970-491-6341; john.volckens@colostate.edu.

Traditional in vitro PM exposure models, however, have drawbacks that lead many researchers to question their validity and applicability to exposure in vivo. Particles are usually collected on a solid matrix (i.e., a filter), extracted and reconstituted in medium, and then delivered to a cell culture. Particle extraction may involve harsh solvents, is rarely 100% efficient, and introduces biases due to particle agglomeration, dissolution, or reaction. Delivery of particles suspended in liquid to respiratory tract cells also makes it difficult to compare the dose of particles on the cells to the particle dose seen by cells during inhalation, sometimes resulting in doses delivered in vitro that may be orders of magnitude larger than those studied in vivo. These limitations, when taken together, make traditional in vitro exposure methods unrepresentative of actual conditions encountered in vivo (2).

However, several advances have emerged in recent years to address these drawbacks. The ability to grow human primary airway epithelial cells on a semipermiable membrane (e.g. Transwells), provides an opportunity to develop exposure systems in which airborne particles can be deposited directly onto the apical cell surface, mimicking in vivo particle deposition. Early designs of direct, air-to-cell deposition systems flowed aerosol above air-liquid interface cultures, allowing for particle deposition by gravitational and diffusive mechanisms. These systems exhibited poor overall deposition efficiencies that depended on flow-rate, chamber geometry, and particle size (3-6). Subsequent designs employed electrical deposition to force charged particles directly onto cell surfaces with applied electric fields (7-9). In this work, we modified the electrostatic deposition system of de Bruijne (6) for increased cell capacity and aerosol flowrate, along with direct heating and humidification of the aerosol stream (described below).

We hypothesize that cellular response to PM depends strongly on the culture system employed, along with the particulate dispersion state (i.e., the mode of delivery to cells). To test our hypothesis, we compared the levels of inflammatory biomarkers expressed by human primary bronchial epithelial cells when exposed to coarse ambient PM using either a traditional delivery system or a delivery system in which airborne particles are deposited directly onto the apical surface of particles grown at an air-interface. Hereafter, we refer to these models as the indirect-liquid and direct-air systems, respectively. Examples of both the direct-air and indirect-liquid culture systems are shown in Figure 1.

Experimental

Cell Culture

The cell and culture systems have been described elsewhere (10). Briefly, normal human bronchial epithelial (NHBE) cells obtained by brush biopsy from three healthy volunteers were expanded in number by culturing them on plastic Petri dishes with epithelial growth medium (BEBM Kit, Lonza Scientific). After 3 passages, cells were seeded onto collagen-coated porous membranes (Snapwell, 1.12 cm^2 area, Corning Life Science, Inc.) for direct-air exposures or 12-well Petri dishes (Costar, 3.8 cm^2 area, Corning Life Science, Inc.) for indirect exposures. After the cells reached confluence, direct-air cultures were supplemented with 500 nM retinoic acid to stimulate differentiation. At 72 hours, the supplemental retinoic acid was lowered to 50 nM and the direct-air culture was taken to an air-interface by removal of the apical medium above cells. Cultures were maintained for three days prior to exposure, as transcriptional profiling indicates that gene expression levels begin to stabilize at day 3 following the change from liquid to air-interface (10). Cultures for indirect, liquid exposures were carried in similar fashion, except that they were maintained submersed in medium and not transitioned to an air-liquid interface.

In vitro Model

The in vitro exposure model, shown in Figure 2, is a modified version of the Electrostatic Aerosol In vitro Exposure System (EAVES) described by deBruinje et al. (7). We modified this system to operate at a higher flowrate, with increased cell capacity and direct heating and humidification of the aerosol stream and cell cultures. The latter two modifications reduce the likelihood of cell injury from dehydration or pH imbalance during exposure. Aerosol flows to the EAVES at a rate of 3.8 L min^{-1} and is first supplemented with 5% CO_2 (Scott Specialty Gasses, Plumsteadville, Pa) to maintain cells at physiological pH. The total flow, 4 L min^{-1}, is heated to 37 °C and humidified to 85% RH using a syringe pump (PHD 22/200, Harvard Apparatus, Holliston, MA) connected to an aerosol conditioning tube similar to the design of Hering (11). The permeation tube allows water vapor to merge with the aerosol stream without appreciable particle losses or dilution. Heated, humidified aerosol is then passed through a unipolar corona charger operating at 10 μA, which renders the aerosol with a net positive charge. Positively charged particles flow into the precipitation zone where a 1.5 kV electric field (repeller plate in Figure 2) forces them directly onto cells. The cell culture plate is made of 100% titanium and machined to hold up to nine air-liquid interface wells (Snapwell, Corning Life Sciences). The corona and repeller plates are independently heated from above and the culture plate is heated from below by a thin polyimide resistor element (Kapton Heat Film, Mcmaster Carr, Elmhurst, IL). Temperature control is maintained to the nearest 0.1 °C with a thermistor-PID control relay (CN1504, Omega Engineering, Stamford, CT) for each zone.

One potential drawback of this system is that the corona charging process releases ozone into the particle flow at a concentration of approximately 80 ppb. Ozone is a strong oxidant known to exacerbate the effects of PM air pollution in both healthy individuals and those with pre-existing airway disease (12,13). However, the levels seen here are not expected to generate significant levels of pro-inflammatory markers in NHBE cells (14). deBruijne et al. reported that air-interface A549 cultures placed in the original EAVES showed no signs of inflammation or stress, despite the exposure to ozone or an applied electric field (7). Because of the modifications to the original EAVES design and the use of primary human cells, we tested both particle collection efficiency and cell viability prior to the exposure experiments. Coarse PM collection efficiency was evaluated with an Aerodynamic Particle Sizer (APS 3321, TSI, Inc.) using a previously-described protocol with NaCl aerosol (15). Cell viability was tested by measuring the time-dependent release of lactate dehydrogenase (CytoTox 96, Promega, Madison, WI) from cultures exposed to particle-free air in the EAVES system operating with corona current and precipitation voltage on. Release of LDH was measured by rinsing the apical compartment (immediately above cells) with phosphate-buffered saline, aspirating the rinse, and combining each into a 1.5 mL centrifuge tube. The release of LDH was measured relative to total cellular LDH (quantified by cell lysis with Triton X) and exposed cultures were compared to controls maintained in an incubator for equal time. We also evaluated the aerosol deposition rate to culture wells by sampling ammonium fluorescein aerosol with the modified EAVES along with a reference filter sampling in parallel (16,17). Fluorescence levels were quantified using a microplate reader (model FL800, Biotek Instruments); blanks were carried for each measurement and a standard, 5-point calibration curve was used to confirm linearity of instrument response.

Direct-Air Exposures

Coarse ambient PM (defined as having aerodynamic particle diameters between 2.5 and 10 μm) from above the EPA Human Studies Facility in Chapel Hill, NC was drawn through a rooftop inlet equipped with a 10 μm size-selective inlet and passed through virtual-impactor system designed to concentrate the coarse fraction 15-20 fold (18). Upon exiting the concentrator, aerosol was sampled isokinetically onto pre-weighed, 47 mm Teflon (Teflo 47 mm, Pall Corp., Ann Arbor, MI) filters for gravimetric analysis. Filters were maintained in a

temperature and humidity controlled environment for at least 12 hours prior to weighing on an analytical microbalance (XP2U, Mettler Toledo, Columbus, OH).

Coarse, concentrated aerosol was drawn at 200 L min^{-1} into a 3.6 m^3 chamber that housed the modified EAVES unit. The exposure duration was constant at 3 hours per test; however, the deposited mass varied with ambient conditions each day, producing some variation in the dose delivered to cells. Cells from three different donors were exposed to PM (3 wells per experiment) with one repetition per donor for a total of six experimental runs. Control cells were exposed to filtered, ambient air with the EAVES chamber running at the same operating conditions as during the exposure tests.

Indirect Liquid Exposures

Ambient, coarse PM was collected onto pre-cleaned, polyurethane foam substrates using a ChemVol High Volume Cascade Impactor (Rupprecht and Patashnick Co, Inc. Albany, NY) as previously described (19). A single batch (1-week integrated sample) was processed and applied for all experiments. Substrates were stored at -80 °C until extraction by sonication in endotoxin-free water for 1 hour . The extracted material was quickly frozen, dried and concentrated by lyophilization, and then stored at -80 °C. Extracts were reconstituted in sterile distilled water to a final concentration of 5 mg mL^{-1}. Reconstituted extracts were then diluted in media, sonicated and vortexed for 1 minute each, and then applied to cells via pipette in a 1 mL bolus.

Gene Expression Analysis

Because air pollution particles can interact with inflammatory proteins produced by epithelial cells, making it difficult to quantify their levels, we chose to quantify levels of mRNA which code for these proteins. Levels of mRNA were measured 1 hour following the exposure for markers of cellular inflammation expressed by three genes of interest: heme-oxygenase-1 (HOX-1), cyclooxygenase-2 (COX-2), and interlukin-8 (IL-8). Total RNA was isolated from cells using a standard protocol (RNeasy Mini Kit, Qiagen) and quantified by spectrophotometry at 260/280 nm. Transcripts were stored at -80 °C and subsequently converted to cDNA prior to quantification with an Applied Biosystems 7500 Real-Time PCR System (TaQMan Cleavage Assay). The Livak ΔΔCt method was used to normalize copy numbers with gylceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. The following primer and probe sets were synthesized by Integrated DNA Technologies (Coralville, IA) for the RT-RT PCR analysis [sense, anti-sense, probe]: *COX-2* [GAATCATTCACCAGGCAAATTG; TCTGTACTGCGGGTGGAACA; TCCTACCACCAGCAACCCTGCCA]; *HOX-1* [CAGCAACAAAGTGCAAGATTCTG; AGTGTAAGGACCCATCGGAGAAG; AGGGAAGCCCCCACTCAACACCC]; *IL-8* [TTGGCAGCCTTCCTGATTTC; TATGCACTGACATCTAAGTTCTTTAGCA; CCTTGGCAAAACTGCACCTTCACACA]; *GAPDH* [GAAGGTGAAGGTCGGAGTC; GAAGATGGTGATGGGATTTC; CAAGCTTCCCGTTCTCAGCC].

Results and Discussion

Particle Collection and Deposition Efficiency

Collection efficiency in the modified EAVES varied from 88% at 0.5 μm to 100% at 10 μm aerodynamic diameter, as shown in Figure 3. In the coarse particle range, however, the average collection efficiency was 98%, indicating that coarse PM was effectively removed by electrical charging and precipitation. The particle collection zone spans an area of 55.3 cm^2, whereas the growth area for each cell insert is approximately 1.12 cm^2. Assuming a uniform deposition rate, each insert could receive a maximum of 2% of the sampled aerosol mass. Deposition tests with ammonium fluorescein particles, however, revealed that each well received only 1.3% of

the sampled aerosol mass. This discrepancy is not surprising, as some of the aerosol is lost to the walls and corona collector plate during charging. The coefficient of variation in deposited mass between wells was approximately 30%, with slightly more mass collecting down the flow centerline. These differences, however, were not statistically significant among replicate tests.

Cell Viability

Although our device attempts to replicate the optimal temperature, humidity, and CO_2 levels required for cell viability, there is a concern that cells with no medium covering them might become dehydrated when exposed to a moving airstream for several hours. To monitor cell viability and cytotoxicity, we measured the release of lactate dehydrogenase (LDH) from the cells into the apical compartment. LDH is a commonly used marker of membrane integrity and serves as a proxy for viability and cytotoxicity. Cells were exposed to particle-free air for 3 hrs with the corona and repeller plate active, thus subjecting them to flowing air and potential agents such as ozone, but not depositing appreciable levels of particles on the cells. Apical release of LDH is shown in Figure 4 for air-interface cultures placed in the modified EAVES sampling particle-free air for 30 to 180 minutes. The 3-day cultures show slightly higher levels of LDH release at 30 and 180 minutes, however, these levels are not substantially higher than basal levels (i.e., incubator controls), nor are they statistically different from the control cultures when compared by t-test ($p > 0.2$). The 21-day cultures show no differences between exposures and controls. Taken together, these results indicate that the modified EAVES system does not appreciably harm cells undergoing clean-air exposures, similar to the findings by de Bruijne et al (7).

Pro-Inflammatory Effects of Coarse PM

Levels of mRNAs coding for inflammatory and oxidatant stress proteins are shown in Figure 5. Each mRNA value is expressed as a ratio of the response of PM-exposed cells divided by the response of air-exposed control cells. For standardization, we report exposure concentrations in terms of delivered mass (μg) normalized to cell surface area (cm^2). As expected, the majority of these ratios are greater than unity, indicating that cells exposed to coarse ambient particulate matter undergo inflammation and oxidative stress. For cells exposed using the indirect-liquid method, a dose-response is somewhat evident. Increasing coarse PM concentrations leads to a statistically significant increase in expression for each gene of interest, as seen in Figure 5. At 7 $\mu g/cm^2$ the median ratios of exposed-cell to control-cell expression of IL-8, HOX-1, and COX-2 were 1.1, 1.0, and 1.5, respectively, with only COX-2 being statistically significant ($p < 0.01$). These ratios increased to 3.3, 1.8, and 2.4 at 65 $\mu g/cm^2$, with all exposures being significantly higher than controls ($p < 0.01$). The variability in mRNA response is likely due to the inter-individual differences typical with primary cell cultures (i.e., phenotype effects) and the relatively small sample size.

For air-interface cells exposed directly to coarse PM using the modified EAVES, mRNA accumulation is also statistically significant. Increases in IL-8, HOX-1, and COX-2 mRNA expression were 1.29, 4.77, and 2.21, respectively, with the increase in IL-8 expression being marginally significant ($p < 0.05$) and HOX-1 and COX-2 being highly significant ($p < 0.01$). More importantly, however, is that the cellular responses from direct, particle-to-cell deposition occur at mass loadings that over an order of magnitude lower than from indirect exposures. This is evident in Figure 5b and 5c, where 2 μg delivered by direct-air deposition produces the same level of HOX-1 and COX-2 transcripts, respectively, as 65 μg delivered by the indirect-liquid method. Levels of mRNA expression are slightly more variable in the direct-air system (as compared to the indirect-liquid system). The increased variability is likely due to daily variation in ambient PM concentrations, leading to slightly different mass loadings for each of the six exposure days (1.5 to 2.5 $\mu g/cm^2$, average ~ 2.0 $\mu g/cm^2$). Because the exposure duration was constant (3 hours), deposited mass levels did not differ enough to effect a dose-

response curve; we chose not to vary the aerosol dilution ratio (to affect variable mass loadings), so as to achieve a sufficient level of experimental replicates for the results presented in Figure 4. Even with the single range shown, the pro-inflammatory effects resulting from direct-air exposures are striking. Interestingly, the accumulation of IL-8 in PM-exposed cultures using the direct-air system is somewhat attenuated relative to the indirect-liquid system (Figure 5a), especially in light of the relative expression of HOX-1 and COX-2 between systems. The signal transduction and transcription factor pathways controlling the expression of IL-8, COX2, and HOX1 are not identical (i.e., it is not necessarily expected that they would all respond the same way to a single toxicant). Differences in particle chemistry or cellular physiology between exposure systems will undoubtedly affect the relative levels of response of these three genes in a manner that is difficult to predict. However, the demonstration that effects are evident at much lower particle concentrations in one system versus the other remains an important result of this work.

Several factors likely contribute to the difference in exposure-response between the direct and indirect methods. Collecting particles on a solid matrix with relatively high air velocity can result in the evaporation of volatile and semi-volatile species, such as quinones and polyaromatic hydrocarbons, which are known to cause cell injury (20). The processes of extraction, lyophilization, and resuspension almost certainly results in several physiochemical changes to the aerosol which might alter its toxicological properties. For example, water soluble components released from particles during extraction may be bound by proteins, anti-oxidants, and other substances found in tissue culture medium, thus reducing or eliminating their toxicity. Additionally, cells contain surface molecules that interact with external stimuli and drive intracellular responses. Soluble compounds may interact differently with such receptors than would particle-bound species, thus potentially activating cellular processes that would not normally become activated by particles alone. Non-polar organic compounds may be released from particles during the sonication process and may never come into contact with the cells due to poor solubility. Even particles that come into contact with cells will react with substances found in tissue culture medium that can bind to and deactive many toxic compounds on their surface. Finally, the process of extracting particles from a solid matrix results in particle agglomeration, reducing the surface area available for reaction with cells.

A major limitation of traditional in vitro particle exposure studies is the difficulty in defining the dose of toxicant delivered to cells when particles are suspended in solution above a submerged culture. With indirect-liquid exposures (Figure 1b), a particle hydrosol suspended in 1 mL of media above a 1.12 cm^2 culture results is an aqueous particle layer approximately 9.8 mm thick. This layer is hundreds of times larger than the cell layer itself, which is on the order of 10-50 μm. Only particles that dissolve, settle, or diffuse to the bottom can potentially interact with cells, and the latter two mechanisms are functions of particle size (which may have changed during particle processing). Because these artifacts are related to particle size, solubility, and composition, many in vitro tests lack comparability, as the determination of a true ‘deposited dose’ is extremely difficult (21). The exposure system described here (Figure 1a and 2) in which intact particles from the air are deposited directly onto the apical surface of cells (as happens when particles are inhaled) allows for a much more accurate assessment of particle dose, making it possible to compare the response of cultured airway epithelial cells following in vitro exposure with the response of airway epithelial cells following in vivo exposure to the same toxicant. We note that a direct comparison between culture systems (i.e., exposure of air-interface cells to PM suspended in culture medium), while desirable, is simply not feasible. A liquid layer added above air-interface cells dramatically alters the oxygen tension at the tissue surface (i.e., a reduction in pO_2 due to reduced O_2 solubility in the liquid medium) and this transformation causes the cells to enter a state of hypoxic stress regardless of whether particles are present in solution or not (unpublished data).

The use of unipolar corona charging may have contributed to the increased inflammatory response seen in direct-air exposed cells. In this system, particles deposited onto cells are positively charged (vs. a Boltzmann-like charge distribution normally found in the atmosphere). Whether positively-charged PM is more toxic than normal ambient PM is unknown. Some transfection agents contain positively-charged amino acids, which appear to enhance cellular uptake (22). Positive charge may also enhance particle transport to cells expressing a net-negative membrane voltage. However, it is also likely that particles become quickly neutralized upon immersion in the extracellular matrix, as the charge on these particles complexes with free ions or is conducted to ground. Additional work is necessary to investigate this phenomenon further. However, we note that another direct-air exposure system described by Holder et al. produced similar results for diesel exhaust aerosol and their system used only gravity and diffusive deposition mechanisms in the absence of applied electrostatics (23).

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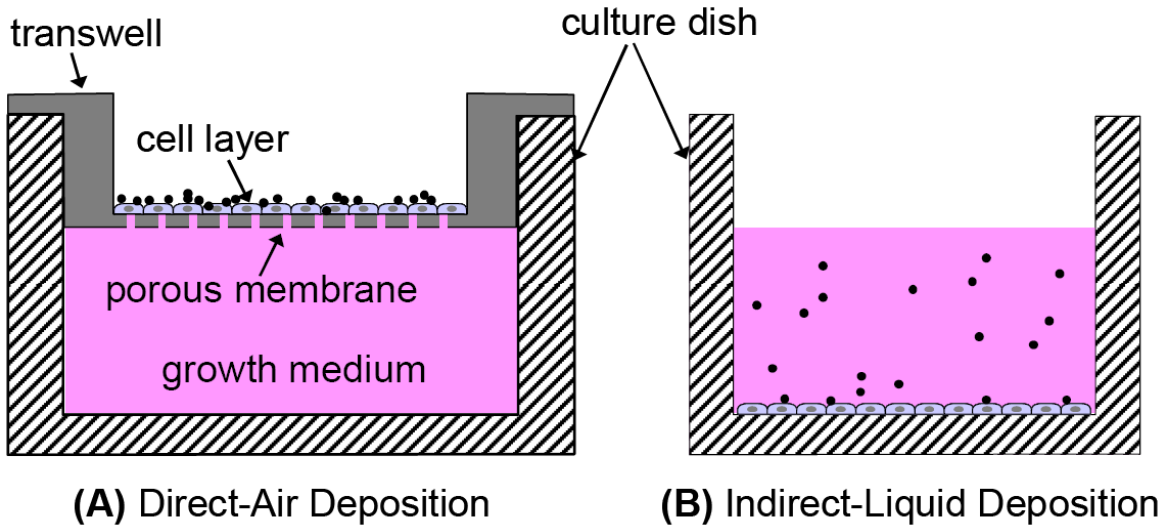

Figure 1.
Diagram of the cell models used in this study. (A) Direct-air deposition, where particles are deposited directly to cells grown at air-liquid interface using electrostatic force (B) Indirect-liquid deposition, where collected particles are resuspended in growth medium and delivered to submersed cells grown on plastic. Note that cell layer and particle sizes are not drawn to scale.

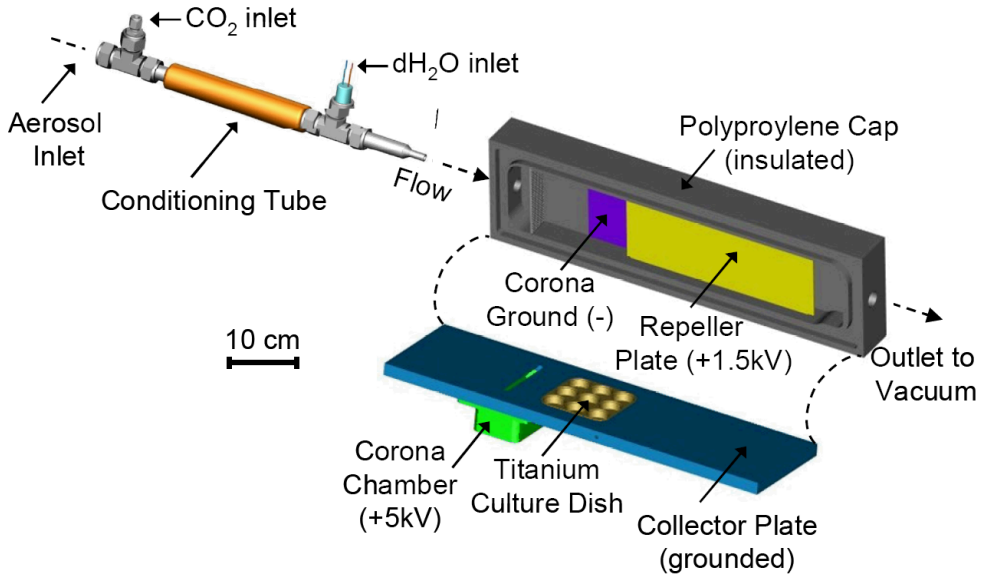

Figure 2.
Schematic of the modified electrostatic aerosol in vitro exposure system (EAVES). The cap is shown in the open position to reveal the repeller plate and culture dish inside.

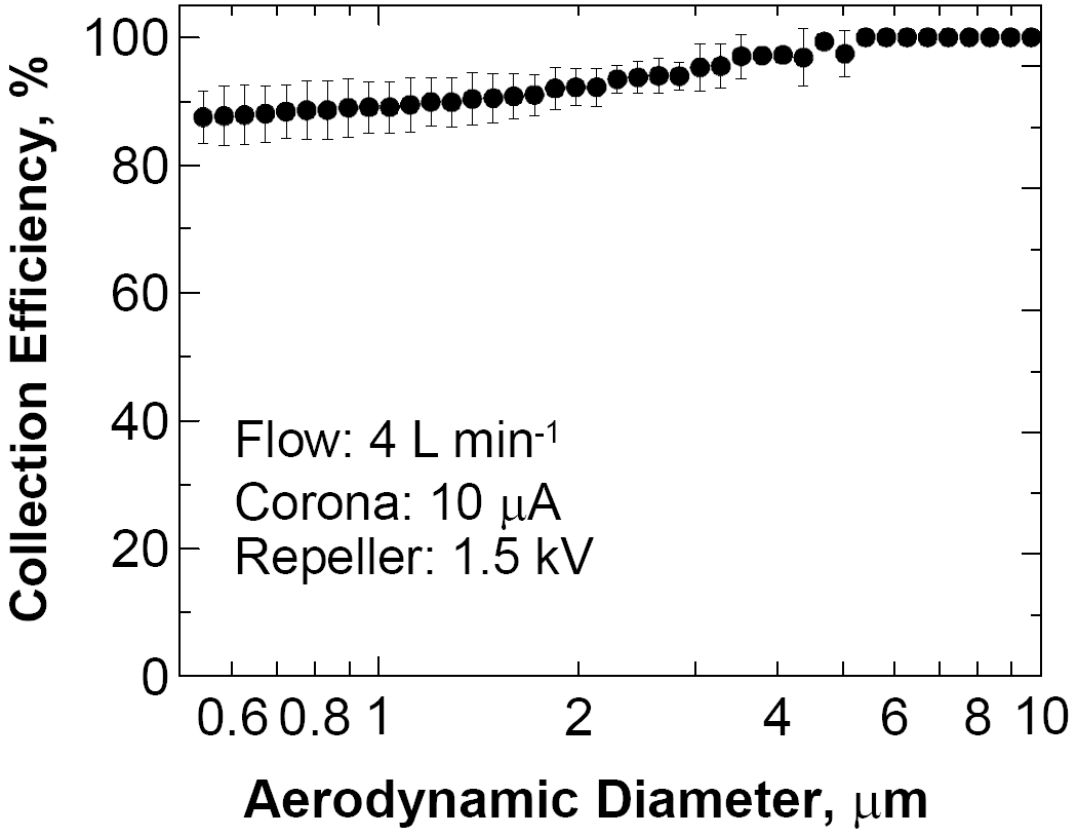

Figure 3.
Collection efficiency by the modified EAVES chamber as a function of aerodynamic particle diameter (NaCl aerosol). Total flow was 4 L min^{-1}, corona current was 10 μA, and repeller voltage was 1.5 kV. Error bars represent one standard deviation; those that are not shown are within the size of the data point.

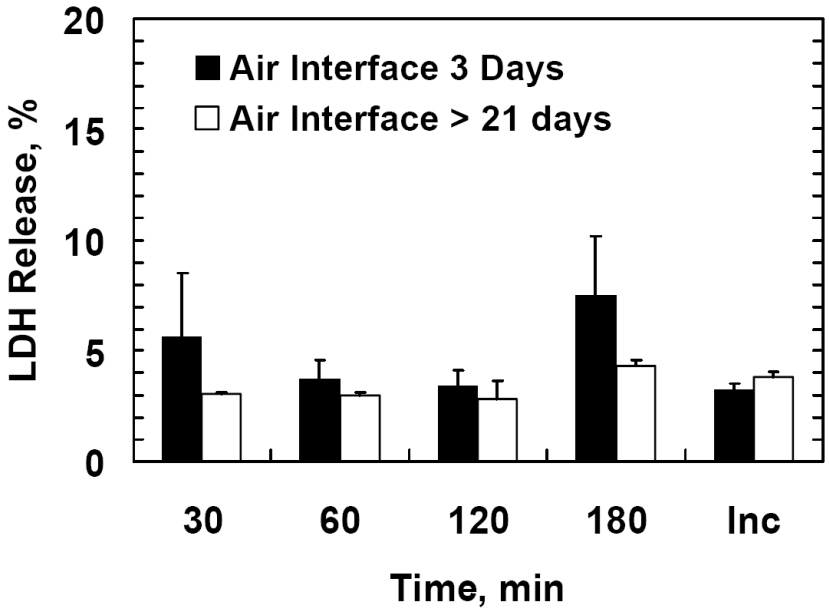

Figure 4.
Time-dependent release of lactate dehydrogenase (LDH) from NHBE cells grown at an air-interface and exposed to particle-free air within the modified EAVES chamber. Incubator controls (Inc) are shown at right. Error bars represent one standard deviation.

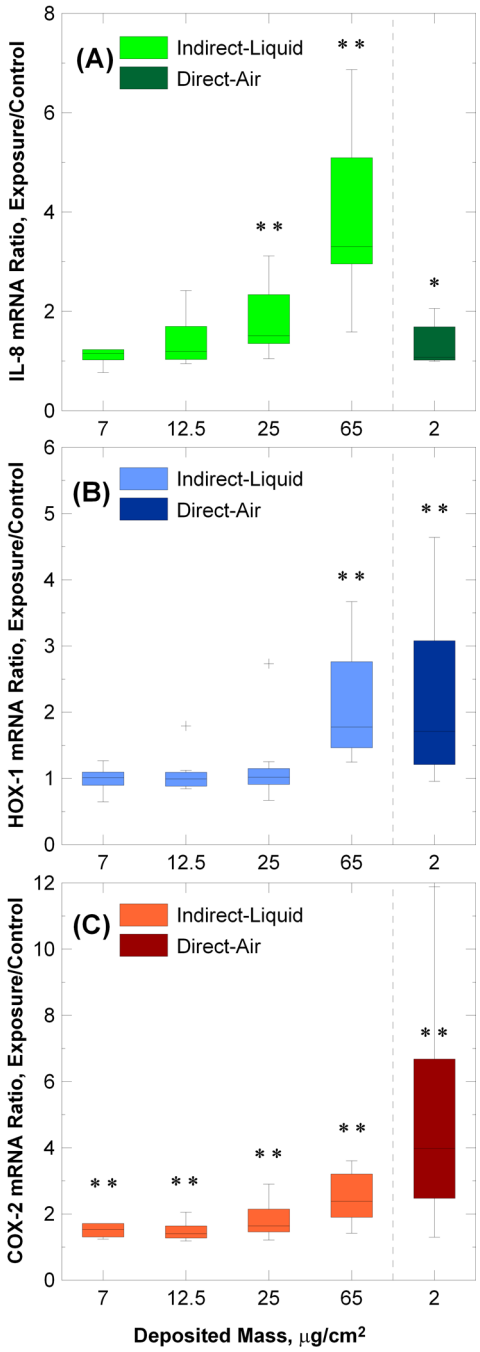

Figure 5.

Box-whisker plots of mRNA expression profiles (ratio of exposure to control) at 1-hour post exposure. Light-shaded boxes represent cells grown submersed and exposed to liquid extracts of PM collected on filters. Dark boxes represent cells grown at an air-interface and exposed directly to coarse PM using the modified EAVES system. The (+) symbols indicate data outliers, the (*) indicates significance at $p < 0.05$ and (**) at $p < 0.01$.