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A New Portable In Vitro Exposure Cassette for Aerosol Sampling

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Dear Dr. Troyer,

Enclosed please find a manuscript entitled "A New Portable In Vitro Exposure Cassette for Aerosol Sampling" (Authors: Lynn E. Secondo, Nathaniel J. Wygal, and Nastassja A. Lewinski) for your consideration as an article in the Journal of Visualized Experiments. All authors have read and approved the submission of the manuscript before exclusively submitting it to this journal. This paper has not been previously published or accepted for publication.

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In this manuscript, we present a new approach for *in vitro* lung toxicity testing that is enabled by the portable *in vitro* exposure cassette or the PIVEC. To our knowledge, this is the first device of its kind to allow for air-liquid interfaced cell exposures to aerosols within the breathing zone of a person. The PIVEC was designed as an adapter to the 37 mm filter cassette that is commonly used to collect aerosols on a filter. By replacing the filter with a framework to support a cell culture insert, the design is capable of exposing cells at the air-liquid interface (ALI). *In vitro* studies utilizing cells grown and exposed at the ALI have been repeatedly demonstrated to provide a more biologically accurate response.

In addition to describing the design, characterization of the PIVEC was performed by measurement of the deposition efficiency, relating the amount deposited onto the cells to the amount administered to the system, over three sizes of copper nanoparticles. Exposure of cells within the PIVEC to the copper nanoparticles was performed with a focus on cytotoxicity and oxidative stress endpoints. The PIVEC is compared to literature values for commercial systems with the same aerosol flow pattern including the Cultex CG and RFS and the Vitrocell. The portability of the PIVEC allows on-site *in vitro* testing of aerosols by taking the lab to the field to investigate near the source of emission. As an inexpensive device, multiple PIVECs can be used to monitor spatial differences. This portability will provide many opportunities that were not previously available with current ALI exposure systems.

If we can provide any further information that would be helpful in evaluating this manuscript, please contact us at (804) 828-0452 or by email at nalewinski@vcu.edu.

Thank you for your time and consideration.

Sincerely,

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TITLE:

A New Portable *In Vitro* Exposure Cassette for Aerosol Sampling

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KEYWORDS:

Exposure system, Inhalation, Nanoparticle, Toxicology, Deposition efficiency, Personal monitoring

SUMMARY:

Here, we present a protocol to perform portable cellular aerosol exposures and measure cellular response. The method uses cells, grown at the air-liquid interface, mimicking *in vivo* physiology. Cellular response to copper nanoparticle aerosols was observed as oxidative stress through reactive oxygen species generation and cytotoxicity as lactate dehydrogenase release.

ABSTRACT:

This protocol introduces a new *in vitro* exposure system, capable of being worn, including its characterization and performance. Air-liquid interface (ALI) *in vitro* exposure systems are often large and bulky, making transport to the field and operation at the source of emission or within the breathing zone difficult. Through miniaturization of these systems, the lab can be brought to the field, expediting processing time and providing a more appropriate exposure method that does not alter the aerosol prior to contacting the cells. The Portable *In vitro* Exposure Cassette (PIVEC) adapts a 37 mm filter cassette to allow for *in vitro* toxicity testing outside of a traditional laboratory setting. The PIVEC was characterized using three sizes of copper nanoparticles to determine deposition efficiency based on gravimetric and particle number concentration analysis. Initial cytotoxicity experiments were performed with exposed lung cells to determine the ability of the system to deposit particles while maintaining cell viability. The PIVEC provides a similar or increased deposition efficiency when comparing to available perpendicular flow *in vitro* exposure devices. Despite the lower sample throughput, the small size gives some advantages to the current *in vitro* ALI exposure systems. These include the ability to be worn for personal monitoring, mobility from the laboratory to the source of emission, and the option to set-up multiple systems for spatial resolution while maintaining a lower user cost. The PIVEC is a

system capable of collecting aerosols in the field and within the breathing zone onto an air-interfaced, *in vitro* model.

INTRODUCTION:

Personal sampling using *in vitro* techniques could provide comprehensive information regarding the biological effects of aerosols in the workplace.¹ Exposures to contaminants in the air include exposures to the chemical itself, to the collected air samples, under submerged conditions where the gas is introduced to the cell suspension, intermittent exposures using a device such as a rocker, or direct exposures at the air-liquid interface (ALI).² Many of these techniques are performed with cells grown in suspension or the collection of samples prior to the exposure, each of which can affect the toxicological study due to potential changes in the aerosol.³ To avoid these changes, the laboratory can be brought to the field using several *in vitro* ALI culture exposure systems that are used in literature,⁴⁻¹³ however, few are commercially available.^{8,9,12} These systems are often bulky, especially when including instruments to regulate the temperature and humidity of the cellular environment and the flow rate of the sample aerosol. By using the PIVEC, aerosol exposures can be performed outside of a traditional lab setting or within the breathing zone while mimicking inhalation conditions.

The determination of aerosol deposition *in vitro* is important to the investigation of health effects due to inhalation. The breathing zone, the area within 30 cm from the mouth and nose,¹⁴ is crucial for understanding the exposure to nanoparticles and for linking to the biological effects in the lungs.² Often, the deposition on cells is defined as a deposition efficiency, the particles deposited onto and taken up by the cells divided by the particles administered to the system^{6,15} or on a mass basis of the same amounts.^{4,16} The current methods for measuring aerosols in the breathing zone are filter based, capturing particles over a given sampling period and using the filters to conduct further testing.¹⁷ Personal monitoring requires a small system that comes with the tradeoff of fewer samples.

There are many approaches to determine the health effects from exposure to an aerosol. The ALI model allows for the aerosol to be administered directly to the cells through the air as in a real exposure scenario, yet it is more cost-effective and less time intensive than *in vivo* studies while mimicking the air-liquid barriers such as the eyes, skin, and lungs. Lung cells grown at the ALI have the ability to generate a polarized barrier layer,^{18,19} which produces physiological traits that resemble the *in vivo* lung epithelium, including mucus and surfactant production in specific bronchial or alveolar cell lines, cilia beating,¹⁹ tight junctions,^{19,20} and cell polarization.¹⁸ Changes such as these can affect the cellular response measured in toxicity studies.²¹ In addition, ALI *in vitro* model results are often more sensitive than cells exposed via suspension models²² and are able to model acute *in vivo* inhalation toxicity.^{23,24} Therefore, an ALI exposure system that is able to perform measurements within the breathing zone is a natural next step.

By exposing the cells to aerosol directly at the source of emission, investigation of the effects of all gases, semi-volatile compounds, and particles involved in the mixture occurs. When the mixture is collected on a filter, the gases and volatile compounds are not captured and the whole mixture cannot be investigated. In addition, reconstitution of particles into a powder or a liquid

suspension can lead to the aggregation or particle-fluid interactions, such as dissolution, in liquid suspension.^{25,26} When aerosol particles are added to the liquid, there is a higher potential for agglomeration,^{25,27} formation of a protein corona,²⁸ or interaction with compounds in the liquid, which can affect deposition and influence the biological response.^{29,30}

Exposure at the ALI is based on three main aerosol profiles, cloud settling, parallel flow, and perpendicular flow. Cloud settling, used by the Air-Liquid Interface Cell Exposure (ALICE),⁴ is a batch system where particles deposit through gravitational and diffusional settling as the aerosol is treated as one unit. Parallel flow, used by the Electrostatic Aerosol *in vitro* Exposure System (EAVES)⁵ and Multiculture Exposure Chamber (MEC) II,⁶ allows for deposition through the addition of Brownian motion through the flow profile. Perpendicular flow, used by a microsprayer,⁷ Nano Aerosol Chamber for In-Vitro Toxicity (NACIVT),¹¹ and commercial ALI systems^{8-10,12}, adds the impaction of particles within the deposition region. Many of these exposure systems are large and bulky, requiring excess systems for aerosol pre-conditioning, pumps for flow, or even heating chambers for incubation of cells. This large size decreases the portability of the system. Instead of sampling directly at the source of emission, these systems often have samples brought to the lab or model aerosols generated for analysis. The complexity of the emitted aerosol can be lost in translation from the field to the lab. The PIVEC is smaller than current systems, with an external surface area of approximately 460 cm² and weighing only 60 grams, with thermal and humidity control incorporated into the system allowing for a highly portable device. The decreased size and weight allow the system to be worn or taken to the source of exposure, permitting direct sampling.

The large size of current exposure systems also decreases the ability to perform sampling to investigate spatial gradients in concentrations. This resolution is key when determining toxicological effects of many potential environmental and occupational hazards such as vehicular exhaust particulate matter or workplace activities where aerosolization occurs. Immediately post-emission, there becomes a spatial variance in particle concentration. This grows with time as the particles disperse throughout the atmosphere and these effects can change based on the ambient conditions, such as temperature, pressure, wind, and sun. Particles can begin to age and oxidize as well once emitted^{31,32} and dispersal rates are affected by the topography; higher concentrations will be found in canyons and tunnels, where dispersion effects are slowed, and lower concentrations can be found where there is a large area for dispersion.³³ These changes in dispersion rates can have significant effects on human health and can be seen when comparing the number of asthmatic adults living in urban versus in rural settings.³⁴ While many exposure systems provide multiple samples at once, multiple systems are necessary with an abundance of large equipment to perform spatial resolution.

By bringing the lab to the field, the time of analysis can be decreased by using the whole cell as a sensor. Following known biological mechanisms and endpoints can aid in the determination of the aerosol composition and size. Due to slow clearance methods, including mucociliary clearance, phagocytosis, and translocation, these particles are often interacting with cells for approximately days to weeks³ generating oxidative stress, inflammation, and even cell death. These biological endpoints can be the starting points for adverse outcome pathways for

cardiovascular disease or chronic obstructive pulmonary disease. In addition, Wiemenn *et al.* performed an array of *in vitro* assays to compare with literature values for short term *in vivo* inhalation toxicity.³⁵ *In vivo* response was predicted with two of four positive results from testing cytotoxicity via lactate dehydrogenase release, oxidative stress from glutathione reduction and hydrogen peroxide formation and release, and inflammation potential from the tumor necrosis factor alpha gene. Out of ten nanosized metal oxides tested, six tested as active (titanium oxide, zinc oxide, and four different cerium oxide) using exposures *in vitro* with confirmation *in vivo*.

In order to study the effects of aerosols in an occupational setting, our lab developed the PIVEC for exposures in the field. Additionally, the PIVEC can be worn for personal sampling to monitor and investigate inhalation exposure like the 37 mm filter cassette³⁶ or multiple systems can be used to achieve spatial resolution within a given area. In this protocol, the characterization and use of the PIVEC is discussed. After exposure, the biological effects are observed through cytotoxicity assays.

PROTOCOL:

Operators must wear personal protective equipment (e.g. lab coat, gloves, goggles) when performing steps 1, 2, 3, 5, and 6.

1. Preparation of Materials

1.1 Prepare materials for system assembly and exposure to ensure repeatability.

1.1.1 Make sure to use new or 70% ethanol cleaned ¼" inner diameter conductive tubing and ¼" outer diameter connectors for the system assembly.

1.1.2 Store test materials including filters, PIVEC components, tweezers, and particle powders in a well-controlled environment, with respect to the temperature and humidity, for at least 24 h prior to the experiment.

NOTE: The temperature should be near room temperature, approximately 20 °C, with relative humidity less than 35%. This is very important to achieve repeatability between experiments.

1.2.3 Prepare particle counters using isopropanol to clean parts and allow for the system warm-up according to the manufacturer recommendations, including the scanning mobility particle sizer (SMPS) and optical particle sizer (OPS) for measurement.

2. Generation of Dry Aerosol

NOTE: Operators should perform aerosol generation in a fume hood.

2.1 Assemble a system to generate dry aerosols

NOTE: The suspension of particles in gas or liquid should be appropriate for the modeled application and cell culture. The following method can be performed using a liquid-based aerosol. The design of the dry aerosol system is from Tiwari *et al.*³⁷ A schematic of the dry dispersal system is shown in **Figure 1**.

2.1.1 Connect the ball valve to each end of the 4" 1/8 size threaded pipe, this will serve as the particle hopper. Connect 2" 1/8 size pipe to one valve.

2.1.2 Weigh copper nanoparticles, in this study the mass concentration for each particle size was kept constant while determining the deposition efficiency. Approximately use 7.5 mg of 40 nm copper nanoparticles, 7 mg of 100 nm copper nanoparticles, and 13 mg of 800 nm copper nanoparticles per exposure. Place copper nanoparticles into the particle hopper through the open end.

NOTE: The amount of copper nanoparticles weighed will serve as the mass based administered concentration.

2.1.3 Place a 3" piece of 1/2" outer diameter (OD) tubing around the 2" pipe and place a HEPA filter inside this short tubing such that the flow direction is through the ball valve.

2.1.4 Connect the vacuum generator to other ball valve using threading. Connect vacuum generator to air tank by placing a 5/16" OD tubing into the push-to-lock connection. Use 1/4" OD tubing to connect the outlet of the vacuum generator to the experimental set-up by placing the tubing over the outlet of the vacuum generator.

2.2 Use of dry aerosol system to generate dry aerosol

2.2.1 Open the air tank by turning the main valve and allow the air flow to the system. Open the valve on the flow regulator on the air tank and set such that the flow through the system is equivalent to the desired settings on the vacuum pump.

2.2.2 Open the ball valve closest to HEPA filter then open the ball valve closest to vacuum generator. Keep these open for approximately 3 s to allow particles to be pulled into the air stream.

2.2.3 Close the ball valve closest to vacuum generator then close ball valve closest to HEPA filter. Allow air from the tank to flow for the duration of the experiment as necessary.

2.2.4 Close main and regulator valves on air tank to stop the flow. Clean ball valves and vacuum generator using 70% ethanol. Autoclave metal pipes for sterilization.

3. Deposition Efficiency Measurement using PIVEC

NOTE: Operators should perform aerosol exposures in a fume hood.

221
222 3.1 Measure the deposition by collecting the copper nanoparticle aerosol generated in step 2.2
223 on a pre-weighed filter. Use the deposited dose, measured using the collected mass on the filter,
224 and the administered dose, measured using the amount of weighed copper particles, to
225 determine the deposition efficiency.
226
227 3.1.1 Keep 1.00 μm pore glass fiber filters under low humidity conditions, described in 1.1.2, for
228 at least 24 h prior to pre-exposure measurements. Weigh an unused filter three times and record
229 the filter weights. Place the unused filter in a cell culture insert.
230
231 3.1.2 Choose appropriate cell culture insert adapter (6 well or 24 well) for PIVEC to support the
232 cell culture insert with the filter. Place the cell culture insert adapter piece on the top of the base
233 of PIVEC, setting into place such that the base of the adapter piece is wider than the top.
234
235 3.1.3 Use tweezers to place filter loaded cell culture insert within adapter piece. Place the top
236 piece on top of adapter piece, settling into place such that the base of the top piece is wider than
237 the top. Wrap PIVEC with a single layer of duct tape.
238
239 3.1.4 Connect 37 mm cassette pieces on top and bottom of PIVEC by pushing into place. Place $\frac{1}{4}$ "
240 barbed adapters into cassette inlet and outlet.
241
242 3.1.5 Wrap the resistive heater around PIVEC such that the wires are at the base. Tape to secure.
243
244 3.1.6 Wrap PIVEC with ~8 rounds of aluminum foil for insulation. Secure with tape.
245
246 3.1.7 Connect 2" long piece of $\frac{1}{2}$ " outer diameter flexible tubing to the adapter on top of PIVEC.
247 Remove porous tubing from sterile water and place within tubing on top of PIVEC.
248
249 3.1.8 Place PIVEC within clamp on the ring stand and secure. Complete set-up with the vacuum
250 pump, particle counters, and aerosol set-up.
251
252 NOTE: The number-based deposited dose can be determined only if particle counters are placed
253 before the PIVEC and after the PIVEC on separate runs.
254
255 3.1.9 Expose filters using step 2.2 of protocol and desired exposure time and flow rates, in this
256 study an exposure time of 10 min at 0.5 LPM was used. Remove PIVEC from the set-up. Take out
257 the cell culture insert and place the exposed filter in filter holder under low humidity conditions
258 for at least 24 h prior to measurements.
259
260 3.1.10 Clean PIVEC with 70% ethanol. Sterilize with ultraviolet light for at least 30 min prior to
261 the next experiment.
262
263 3.1.11 Weigh the exposed filter three times and record the filter weights. Place the exposed filter
264 in a labeled filter holder for storage.

4. Calculation of Deposited Dose and Deposition Efficiency

NOTE: Knowledge of the deposition is important for aerosol administration and interpretation of cellular response.

4.1 Calculate deposition from mass-based measurements

4.1.1 Calculate the deposited mass on filters as the difference between the pre-exposure average weight and post-exposure average weight. This value is the mass-based deposited dose for the experiment.

4.1.2 Use administered mass, m_{admin} , and mass-based deposited dose determined in 4.1.1, m_{dep} , to calculate the mass-based deposition efficiency, η_m , for the experiment.

$$\eta_m = \frac{m_{dep}}{m_{admin}} \times 100\%$$

4.1.3 Average values from 4.1.1 and 4.1.2 for at least 3 experiments to determine deposition and deposition efficiency for PIVC for particle size.

4.2 Calculate deposition from number-based measurements

4.2.1 Ensure that measurements with particle counters have been performed with counters after the PIVC and to determine the particle concentration before the PIVC. Integrate the particle concentration over time for the particle counter then integrate over particle diameter to determine the total particles measured.

4.2.2 Calculate the deposited particle number as the difference between the particles administered and the particles measured post-PIVC. This value is the number-based deposited dose for the experiment.

4.2.3 Use administered particles, n_{admin} , number-based deposited dose, n_{dep} , volumetric flow rate, V , and time, t , to calculate the number-based deposition efficiency, η_n , for the experiment.

$$\eta_n = \frac{n_{dep}}{n_{admin} V t} \times 100\%$$

4.2.3 Average values from 4.2.2 and 4.2.3 for at least 3 experiments to determine deposition and deposition efficiency for PIVC for particle size.

5. Aerosol Exposure of Cells

NOTE: For the cell culture at the air-liquid interface the reader is referred to Blank *et al.*³⁸ Operators should perform cell culture insert loading (steps 5.1.2-5.1.4) within a biosafety cabinet. Operators should perform aerosol exposures in a fume hood.

5.1 Culture Cells at Air-Liquid Interface

5.1.1 Lift A549 cells from culture flask by adding trypsin-EDTA, 3 mL for a T75 flask or 1 mL for a T25 flask and incubate for 5 min at 37 °C. Add 7 mL of complete media for a T75 flask or 4 mL of complete media for a T25 flask to flask and rinse flask wall with cell suspension to maximize the recovered cell number. Transfer the cell suspension to a sterile 15 mL conical tube then centrifuge cells at 800 x g for 3 min.

5.1.2 Remove the supernatant containing trypsin-EDTA and resuspend the cell pellet in 10 mL of complete media. Remove 10 µL of cell suspension and add to the hemocytometer. Count cells using a hemocytometer to determine the concentration and the total number of cells.

5.1.3 Place 0.5 mL of complete media to each well within a 24 well plate. Place unused cell culture inserts in wells. Seed cell culture inserts on the apical side at a cell density near 1×10^5 cells/cm² for cell types that grow at a rate near doubling per day. To seed A549 cells within a 24 well insert, seed cells at a density of 1×10^5 cells/cm² by adding 35,000 cells to the cell culture insert.

NOTE: Cells with a slower growth rate can be seeded at an increased cell density.

5.1.4 Add complete media to the apical side of cell culture insert to reach the final volume (for 24 well plate final volume is 0.25 mL).

5.1.5 Culture for 7 days in submerged conditions, replacing media every 1-2 days. After 7 days, remove the apical media and culture for at least 1 day in ALI conditions, replacing only the basolateral media.

5.2 Assemble PIVEC

5.2.1 Allow cells to equilibrate to the air-liquid interface for at least 24 h prior to exposure.

5.2.2 Choose appropriate cell culture insert adapter for PIVEC to support the cell culture insert with the filter. Place cell culture insert adapter piece on top of PIVEC base, setting into place such that the base of the adapter piece is wider than the top. Add 4 mL of cell culture media to the well of the base of the PIVEC.

5.2.3 Use tweezers to place the cell culture insert within adapter piece placed in step 5.2.3. Place top piece on top of adapter piece, settling into place such that the base of the top piece is wider than the top. Carefully, wrap PIVEC with a single layer of duct tape.

5.2.4 Connect 37 mm cassette pieces on top and bottom of PIVEC, by pushing into place. Place ¼" barbed adapters into cassette inlet and outlet.

5.2.5 Wrap resistive heater around PIVEC such that the wires are at the base. Tape to secure.

5.2.6 Wrap PIVEC with ~8 rounds of aluminum foil for insulation. Secure with tape.

5.2.7 Connect short piece of 1/2" outer diameter flexible tubing to the adapter on the top of PIVEC. Remove porous tubing from sterile water and place within tubing on top of PIVEC.

5.2.8 Place PIVEC within clamp on the ring stand and secure. Complete the set-up with the vacuum pump and aerosol set-up.

5.3 Expose Cells at ALI using the PIVEC

5.3.1 Use deposition efficiency determined in Step 2 to calculate the mass of particles to be aerosolized. Weigh appropriate mass and add to the aerosol system set up following step 2 within the fume hood.

5.3.2 Expose cells by following step 2.2, in this study of biological endpoints the cells were exposed to approximately 3.5 mg of copper nanoparticles with a flow rate of 0.5 LPM and an exposure duration of 10 min. Control studies performed used humidified air to determine the influence of the air alone. Remove PIVEC from the set-up. Take out the cell culture insert, place in the sterile well plate and return to the CO₂ incubator (37°C, 5% CO₂, 90% RH).

5.3.3 Aspirate media from PIVEC. If performing additional experiments, rinse bottom of PIVEC with phosphate buffered solution then repeat step 5.1 and 5.2.

5.3.4 Clean PIVEC with 70% ethanol when finished. Sterilize with ultraviolet light for at least 30 min prior to the next experiment.

5.4 Biological Assay Procedures

NOTE: Assays performed in this study were oxidative stress generation through the DCFH-DA assay and cytotoxicity through lactate dehydrogenase (LDH) release.

5.4.1 Dissolve 24.4 mg of DCFH-DA in 50 mL methanol to make 1 mM DCFH-DA solution. This solution can be stored at -20°C for up to 4 months. Dilute 1 mM DCFH-DA solution by mixing 0.1 mL of 1 mM DCFH-DA solution with 9.9 mL HBSS to make 10 mL of 10 µM DCFH-DA.

5.4.2 Remove cell culture media and wash cell culture insert with approximately 1 mL of PBS. Add 0.5 mL of 10 µM DCFH-DA solution to each well, replacing inserts when finished. Cover plate with aluminum foil to prevent photoactivation of the dye and return to 37°C incubator for 1 h.

5.4.3 Remove cells from the incubator and aspirate the DCFH-DA working solution from the wells. Add 0.5 mL HBSS to wells and replace cell culture inserts.

5.4.4 Load the well plate into plate reader and measure baseline fluorescence using

excitation/emission wavelengths of 485/530 nm. Remove plate from plate reader and load insert into PIVEC for exposure.

5.4.5 Expose cells for desired exposure duration. Remove insert from PIVEC and return to well plate. Remove 50 μ L of basolateral fluid from well plate and place in white 96 well plate. Measure the fluorescence of DCF using excitation/emission wavelengths of 485/530 nm every 30 min post-exposure for 2 h.

5.4.6 Let basolateral fluid equilibrate to room temperature for 20-30 min. Add 50 μ L of LDH assay solution, mixed following manufacturer protocol, to basolateral fluid from well plate and let react for 10 min. Add 25 μ L of stop solution to well. Read fluorescence of resorufin product using excitation/emission wavelengths of 560/590 nm.

5.4.7 Remove additional basolateral fluid and repeat step 5.4.6 at 4 h and 24 h post-exposure.

6 Statistical Methods

6.1 Analysis of Biological Assay Data

6.1.1 Report ROS production as the fluorescence intensity increase of treated cells relative to baseline measurements. Report LDH activity as the fluorescence intensity increase of treated cells relative to untreated cells.

6.1.2 Perform single factor ANOVA to determine statistical differences between data sets. Where appropriate, perform student t-tests at a value of significance of 0.05. Report data as the mean \pm standard deviation of at least three exposure measurements.

REPRESENTATIVE RESULTS:

Occupational *in vitro* toxicology involves maintaining cellular viability while performing aerosol exposure. The PIVEC system is shown in **Figure 2**, including the temperature and humidity control and the worn PIVEC. The temperature was maintained using a battery-powered resistive heater and the aerosol humidified using increased natural humidification through a porous, wetted tube. In a controlled aerosol setting inside a laboratory, the PIVEC can be set-up for exposure shown in **Figure 1**. Characterization of the system allows for the determination of the deposited dose onto the cells which can then be correlated to the cellular response.

The deposition efficiency is dependent on the size of particles added to the system since deposition forces include impaction, sedimentation, and diffusion. In addition, the deposition is dependent on flow rate, exposure time, and the characteristics of the exposure system. With this information, the deposition can be predicted. The deposition efficiency of the PIVEC has been determined through two methods, gravimetric analysis, and particle number counting. Equations 1 and 2 were used to determine the deposition efficiencies in **Table 1** using the filter based, scanning mobility particle sizer (SMPS), and optical particle sizer (OPS) measurements, **Figure 3**.

Increased deposition is observed overall for the 24 well design than the 6 well design and slightly decreases for 100 nm in comparison to 40 nm and 800 nm copper particles. The deposition in the 24 wells is very uniform over the insert, however, deposition in the 6 well design is lacking uniformity as most of the particles deposit near the center of the insert. Post-exposure analysis can be expedited by determining the dose relationship between the 37 mm filter and the cell culture insert, decreasing the necessity to determine the dose after cellular exposure. Comparison of the deposition within the 37 mm filter cassette and the PIVEC shows a strong correlation for all sizes and wells with a Pearson correlation of above 0.7, however only for 800 nm is the correlation significant with a $p < 0.05$, see **Figure 4**. Compared to similar systems throughout literature,^{11,12} the deposition efficiency of the PIVEC over the range of particle sizes tested is comparable or increased over reported values, observed in **Figure 5**. The deposition efficiency within the PIVEC can be improved through minimizing losses to the system using electrostatic dissipative or conductive plastic or similar material to design the PIVEC.

If filters are not well dried in a humidity-controlled environment before exposure, excess water increases the initial mass and can produce non-physical, negative deposited doses. Variable flow rates can also promote inconsistent deposited doses within the system. To avoid these issues, allow at least one day prior to and after exposure for filters to dry in a humidity-controlled environment.

Cellular responses will be affected by the deposited dose and may be affected by exposure duration if the cells are not properly maintained. A549 cells, an alveolar epithelial carcinoma cell line, were exposed for 10 min to varying sizes of copper nanoparticles at a flow rate of 0.5 LPM. Alternative perpendicular flow exposure systems use between 0.005 LPM and 1.5 LPM for a sustained exposure period whereas this method uses a moderate flow rate during a rapid exposure. Using the mass-based deposition efficiency measured and administered dose, 1.58 ± 0.04 mg/cm² of 40 nm copper, 0.30 ± 0.00 mg/cm² of 100 nm copper particles, and 0.32 ± 0.01 mg/cm² of 800 nm copper particles were deposited onto the cells. Cytotoxicity and oxidative stress were observed within the first twenty-four hours post-exposure. Cytotoxicity was measured using the release of lactate dehydrogenase (LDH) from damaged cells immediately, 4 hours, and 24 hours post-exposure. There was no significant toxicity from copper nanoparticles below 1.62 mg/cm² within 4 hours of exposure, **Figure 6b**. Twenty-four hours post-exposure there was a statistically significant decrease in cell viability of less than 20% viability for cells exposed to copper nanoparticles. The intracellular oxidative stress was investigated using the DCFH-DA assay through the oxidation of DCFH by reactive oxygen species within the first two hours post-exposure, **Figure 6a**. Significant levels of oxidative stress were measured at thirty minutes post-exposure for cells exposed to copper nanoparticles of all sizes. The level of oxidative stress continued to increase at similar rates for all sizes tested within the two hours observed.

Success of the exposures can be determined through the repeatability of cellular response. Two acceptance criteria proposed for cytotoxicity assays include: 1) the % total LDH leakage for the positive control should be greater than 50%, and 2) the positive and sample replicate coefficient of variations should be within 50%.³⁹ If these criteria are not met, this suggest differences between experiments, such as altered deposition amounts, or poor humidity or temperature

control. In addition, observing cytotoxicity measurements can lead to understanding of the experimental controls and aid in determining the error. When the flow rate is too high, cells may die from high amounts of shear stress. By lowering the flow rate, the stress upon the cells from the flow can be decreased.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic of Dry Dispersal System and Experimental Set-up.

Figure 2. PIVEC Design. A) Full Design Pictured with 30 cm Tall Box for Comparison. B) PIVEC with Temperature and Humidity Control Pictured with 30 cm Tall Box for Comparison. C) Worn PIVEC. D) PIVEC Top Piece. E) Cell Culture Adapter for 24 well (left) and 6 well (right). F) Bottom Piece.

Table 1. PIVEC Deposition Efficiency.

Figure 3. Particle number concentration of copper nanoparticle aerosols. A) SMPS measurement. B) OPS measurement.

Figure 4. Relationship between deposition on cell insert and SKC 37 mm filter. Error ± 0.002 mg. A) 40 nm copper particles. B) 100 nm copper particles. C) 800 nm copper particles.

Figure 5. Deposition Efficiency of PIVEC Compared to Perpendicular Flow Exposure Systems in Literature. Literature Values from Perpendicular Flow Exposure Systems are Plotted in Solid Markers and PIVEC Values are in Empty Markers.

Figure 6. Cellular Response to Copper Nanoparticles Post-Exposure (PE). For all measurements, $n=3$ and $p<0.05$. A) Oxidative Stress determined using the DCFH-DA Assay. B) Cytotoxicity determined using the LDH Assay.

DISCUSSION:

Filter cassettes provide a simple, inexpensive method of collecting aerosols in the breathing zone; however, aerosol samples extracted from filters do not represent the entire aerosol (i.e. gases, volatiles, and particulates) and consequently limit the assessment of related biological effects. Using the initial design of the 37 mm filter cassette, the PIVEC is designed to maintain portability and mimic the *in vivo* deposition of particles from inhalation. The PIVEC is significantly smaller than current ALI exposure systems, approximately the size of a tissue box with temperature and humidity control included. The size is similar to personal cascade impactors while offering data on cellular response to the aerosol in addition. While the PIVEC contains space for one sample in comparison to other current exposure systems, the small size permits multiple systems to be used at once, therefore, increasing the sample size and allowing for spatial distribution to be monitored.

There are several critical steps in the protocol. To determine the deposition efficiency, it is critical to properly condition the filters, as described in Step 1. Additionally, it is important to properly weigh the copper nanoparticles prior to exposure and the filter post exposure to determine the mass-based deposition efficiency. The number deposition efficiency must be determined using the difference in deposition between the initial aerosol concentration and final concentration determined using the particle counters. If the deposition efficiency is not properly determined, it is difficult to determine the biological response differences between aerosol types. Modifications to the deposition include altering the deposition. Deposition can be increased by changing the flow rate and exposure duration. This may also influence the cell viability with the potential of drying out the cells. The conditioning of aerosols is often performed to compensate for physiological attributes such as body temperature and humidification in the airways. Increased humidity, over 50%, mimics inhaled air and decreases cell death due to vehicle exposure.⁴³ When temperature and humidity are not well controlled, the cellular response can be influenced. By decreasing the flow rate, additional particles of all sizes will deposit, increasing the deposition. Exposure duration is proportional to deposition, allowing more particles to deposit over an extended experimental period. Conditioning of the aerosol is important when increasing the exposure duration so that the cells do not dry out which can affect biological responses.

The PIVEC uses perpendicular flow to deposit particles via impaction, sedimentation, and diffusion onto the cells which have the potential to dry out cells through the flow and added stress on the cells. The deposition efficiency is dependent on the size of the particle due to the forces of deposition. Many of the current exposure systems with perpendicular flow have a deposition efficiency of below 10% and much closer to 1%. The PIVEC has a deposition efficiency determined by gravimetric analysis of 3% to 16% for a 24 well cell culture insert. The particle number-based deposition efficiency is approximately 1% to 7% for the same insert. When using a 6 well cell culture inserts, these numbers decrease, with the exception of the smallest particle size, due to the streamlining of the aerosol as more particles are confined in the cell culture insert without space for the flow to develop. The 6 well cell culture inserts permit the aerosol streams to bypass deposition. Other perpendicular flow systems have been characterized on a mass basis using 60 nm fluorescein particles⁴⁰, 80 nm and 180 nm copper particles¹⁶, and 60 nm adipic acid particles⁴¹. The resulting deposition efficiencies are generally between 0.05% and 11%. On a number basis, these systems have been characterized using polystyrene particles^{12,15}, carbon nanoparticles¹², and silica particles⁴², yielding efficiencies between 0.2% and 11%. The PIVEC provides, similar or increased, deposition in comparison to available cellular exposure devices.

Cell exposures were performed using copper nanoparticles of 40 nm, 100 nm, and 800 nm. Cell viability was defined using the LDH assay with no significant decrease in viability within four hours post-exposure. The LDH assay was used with a commercial exposure system for 74 ng/cm² after 2 hours and 148 ng/cm² after 4 hours of 9.2 nm copper oxide particles⁴⁴ and 1 µg/cm² deposited after a sequential exposure 4 hours, incubation for 2 hours, then another exposure for 4 hours of 25 nm copper oxide particles⁴⁰, both measuring significant decreases in cell viability. Cytotoxicity was observed in another system for 1.6-7.6 µg/cm² of 180 nm particles copper nanoparticles¹⁶ measured by trypan blue dye exclusion. Exposures of 15 minutes to 40-80 nm

copper oxide nanoparticles decreased viability, measured 24 hours post exposure. The lower toxicity observed using the PIVEC was likely due to the shorter exposure time of 10 minutes and shorter post exposure measurement times. After four hours post-exposure, viability of cells exposed in the PIVEC decreased. Cells exposed to humid air controls observed no significant cytotoxicity, in agreement with other studies^{9,40,44–46}. The oxidative stress was determined using the DCFH-DA assay. The production of reactive oxygen species, such as hydrogen peroxide or oxygen radicals, generated an amount of stress that can lead to growth arrest or cell death. After the cell exposures, there was a minimal increase in oxidative stress for cells kept in the incubator as control and for cells exposed to humid air. Elevated oxidative stress occurred for all particle sizes, increasing within 30 minutes post-exposure. Within one study, 9.2 nm copper oxide particles⁴⁴ and 25 nm copper oxide particles⁴⁰ also induced oxidative stress measured via the carboxy-DCFH-DA assay. Increased exposure time from 2 hours to 4 hours elevated oxidative stress for 9.2 nm copper oxide particles⁴⁴. After four hours of exposure, the 9.2 nm copper oxide particles produced a similar oxidative response as the sequential exposure of 25 nm copper oxide particles⁴⁰, suggesting that exposure duration has a higher influence on oxidative stress response than particle size. Cells exposed within the PIVEC produced elevated oxidative stress compared to that study, however, the particles exposed in the alternate system are copper oxide and will dissolve less quickly than the copper exposed within the PIVEC. Studies performed on the dissolution of copper based on the composition and size of particles agree that larger particles and the metal oxides release ions slower than metal particles or their nanoparticle counterparts^{16,47–49}. As the humid air controls did not produce significant amounts of oxidative stress compared to the incubator control, the influence of copper particles to induce oxidative stress is consistent within the PIVEC to similar *in vitro* exposure systems. The biological responses observed using the PIVEC suggest that the PIVEC is an appropriate system for cellular exposure.

While the characterization and cellular studies of the PIVEC agree well with literature,^{9,16,40,44,46} the device has limitations. The small design decreases the number of samples that can be exposed simultaneously within a single device in comparison to other exposure systems. Other systems allow for at least three cellular exposures to the same aerosol^{9,12} facilitating replicate measurements. Although the PIVEC only allows for one insert per system, the small size allows for multiple systems to easily be used, helping to mitigate this issue. While other personal monitoring systems do not use cells, the PIVEC must be kept near vertical to reduce spilling the cell culture media necessary to preserve cellular viability. Although the PIVEC has not yet been optimized for specific particle ranges (e.g. PM₁₀, PM_{2.5}, PM_{0.1}), the PIVEC has been characterized for a range of particle sizes. Similar to other perpendicular flow systems, the PIVEC shows a decreased ability to deposit particles near 100 nm in diameter, while 40 nm and 800 nm particles deposited with similar efficiency.

The analysis of cells post-exposure can be expedited by performing a parallel collection of aerosols within the PIVEC and an SKC 37 mm filter cassette. A high correlation of gravimetric based deposition allows for the estimation of the particle mass collected on the cells from data collected using 37 mm filters. Comparison of the insert to the filter cassette reduces the need to collect additional samples and additional measurements to determine the dose. Work in progress includes the integration of a real-time monitoring system for cytotoxicity, oxidative stress, or

other biological endpoints. The small size of the PIVEC allows it to be used in a variety of settings, such as on the body as a personal monitor, on a drone above a chemical plant, or outside in the environment for spatial resolution. This method has shown the use of the PIVEC for the collection of aerosol particles onto cell cultures grown at the ALI. By conditioning the aerosol to 37 ± 1 °C and > 80% relative humidity, cellular viability can be maintained during acute exposures. This method is appropriate for both liquid droplet and solid particle-based aerosols and has been shown to deposit particles between 40 nm and 800 nm in cell culture inserts. The versatility of the PIVEC allows this method to be used in multiple settings with a variety of biological endpoints.

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DISCLOSURES:

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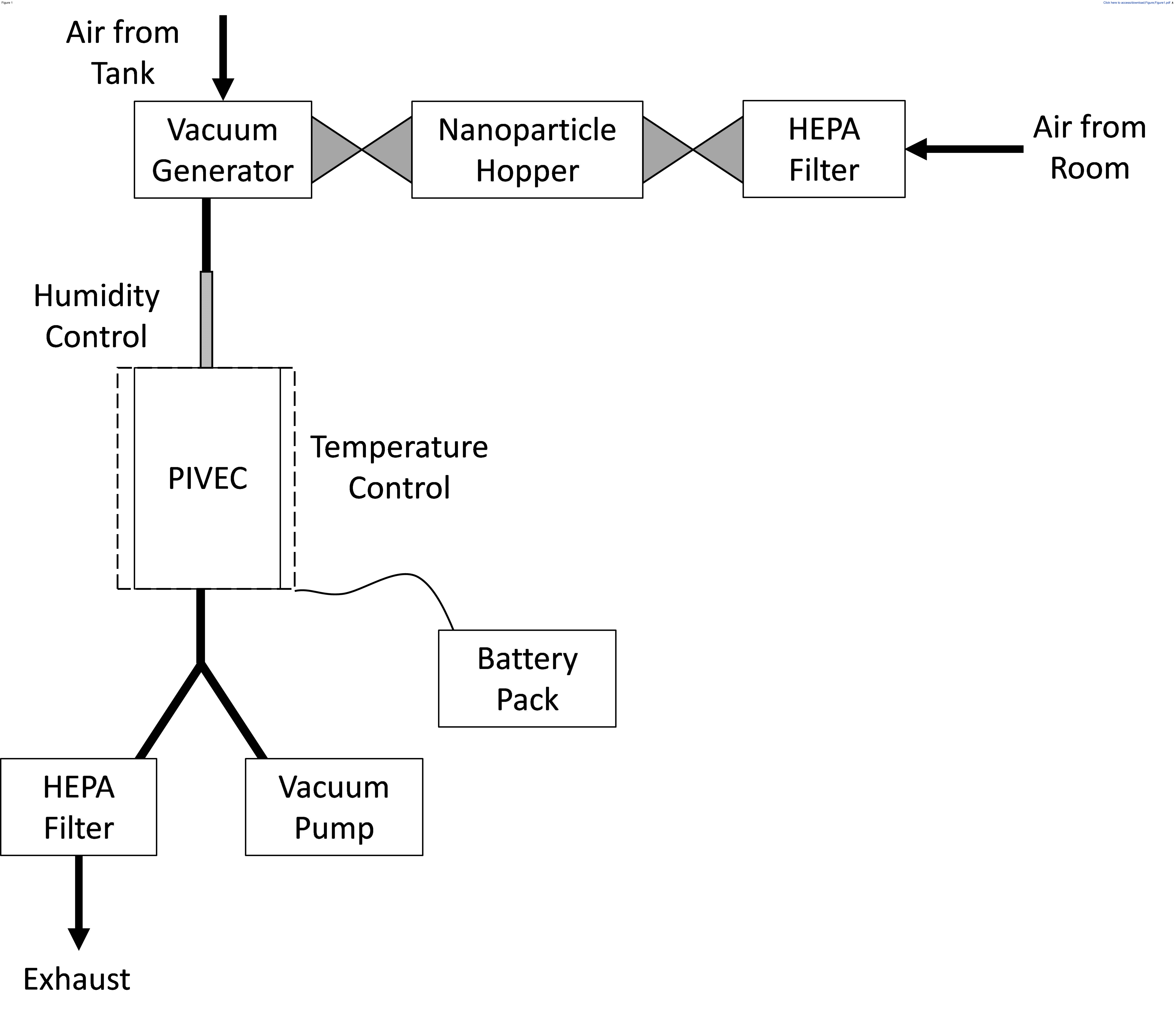
REFERENCES:

1. Lewinski, N. A., Secondo, L. E. & Ferri, J. K. Enabling Real-Time Hazard Assessment at the Workplace Enabling Real-Time Hazard Assessment at the Workplace. *14th Global Congress on Process Safety*. 1–9 (2018).
2. Bakand, S., Winder, C., Khalil, C. & Hayes, A. Toxicity assessment of industrial chemicals and airborne contaminants: transition from in vivo to in vitro test methods: a review. *Inhalation Toxicology*. **17** , 775–787, doi:10.1080/08958370500225240 (2005).
3. Bakand, S. & Hayes, A. Troubleshooting methods for toxicity testing of airborne chemicals in vitro. *Journal of Pharmacological and Toxicological Methods*. **61** (2), 76–85, doi:10.1016/j.vascn.2010.01.010 (2010).
4. Lenz, A. G. *et al.* A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. *Particle and Fibre Toxicology*. **6** , 32, doi:10.1186/1743-8977-6-32 (2009).
5. de Bruijne, K. *et al.* Design and testing of Electrostatic Aerosol in Vitro Exposure System (EAVES): an alternative exposure system for particles. *Inhalation Toxicology*. **21** , 91–101, doi:10.1080/08958370802166035 (2009).
6. Asimakopoulou, A., Daskalos, E., Lewinski, N., Riediker, M., Papaioannou, E. & Konstandopoulos, A. G. Development of a dose-controlled multiculture cell exposure chamber for efficient delivery of airborne and engineered nanoparticles. *Journal of Physics: Conference Series*. **429** , 1–10, doi:10.1088/1742-6596/429/1/012023 (2013).

7. Grigg, J. *et al.* DNA damage of macrophages at an air-tissue interface induced by metal nanoparticles Macrophage. *Nanotoxicology*. **3** (4), 348–354, doi:10.3109/17435390903276917 (2009).
8. Aufderheide, M., Knebel, J. W. & Ritter, D. An improved in vitro model for testing the pulmonary toxicity of complex mixtures such as cigarette smoke. *Experimental and Toxicologic Pathology*. **55**, 51–57, doi:10.1078/0940-2993-00298 (2003).
9. Aufderheide, M., Halter, B., Möhle, N. & Hochrainer, D. The CULTEX RFS: A comprehensive technical approach for the in vitro exposure of airway epithelial cells to the particulate matter at the air-liquid interface. *BioMed Research International*. **2013** (1), 1–15, doi:10.1155/2013/734137 (2013).
10. Tippe, A., Heinzmann, U. & Roth, C. Deposition of fine and ultrafine aerosol particles during exposure at the air/cell interface. *Journal of Aerosol Science*. **33**, 207–218 (2002).
11. Savi, M. *et al.* A novel exposure system for the efficient and controlled deposition of aerosol particles onto cell cultures. *Environmental Science and Technology*. **42**, 5667–5674, doi:10.1021/es703075q (2008).
12. Fröhlich, E. *et al.* Comparison of two in vitro systems to assess cellular effects of nanoparticles-containing aerosols. *Toxicology in Vitro*. **27**, 409–417, doi:10.1016/j.tiv.2012.08.008 (2013).
13. Frijns, E. *et al.* A Novel Exposure System Termed NAVETTA for in Vitro Laminar Flow Electrodeposition of Nanoaerosol and Evaluation of Immune Effects in Human Lung Reporter Cells. *Environmental Science and Technology*. **51** (9), 5259–5269, doi:10.1021/acs.est.7b00493 (2017).
14. Vincent, J. H. *Aerosol Science for Industrial Hygienists*. (Elsevier: 1995).
15. Fujitani, Y., Sugaya, Y., Hashiguchi, M., Furuyama, A., Hirano, S. & Takami, A. Particle deposition efficiency at air–liquid interface of a cell exposure chamber. *Journal of Aerosol Science*. **81**, 90–99, doi:10.1016/j.jaerosci.2014.10.012 (2015).
16. Elihn, K., Cronholm, P., Karlsson, H. L., Midander, K., Odnevall Wallinder, I. & Möller, L. Cellular Dose of Partly Soluble Cu Particle Aerosols at the Air-Liquid Interface Using an In Vitro Lung Cell Exposure System. *Journal of Aerosol Medicine and Pulmonary Drug Delivery*. **26** (2), 84–93, doi:10.1089/jamp.2012.0972 (2013).
17. *Aerosols Handbook Measurement, Dosimetry, and Health Effects*. (CRC Press: Boca Raton, 2005).
18. de Souza Carvalho, C., Daum, N. & Lehr, C. M. Carrier interactions with the biological barriers of the lung: Advanced in vitro models and challenges for pulmonary drug delivery. *Advanced Drug Delivery Reviews*. **75**, 129–140, doi:10.1016/j.addr.2014.05.014 (2014).
19. Fattal, E., Grabowski, N., Mura, S., Vergnaud, J., Tsapis, N. & Hillaireau, H. Lung Toxicity of Biodegradable Nanoparticles. *Journal of Biomedical Nanotechnology*. **10** (10), 2852–2864, doi:10.1166/jbn.2014.1939 (2014).
20. Klein, S. G., Serchi, T., Hoffmann, L., Blömeke, B. & Gutleb, A. C. An improved 3D tetra-culture system mimicking the cellular organisation at the alveolar barrier to study the potential toxic effects of particles on the lung. *Particle and Fibre Toxicology*. **10**, 31, doi:10.1186/1743-8977-10-31 (2013).
21. Oberdörster, G. *et al.* Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. *Particle and Fibre Toxicology*.

- 2 , 8, doi:10.1186/1743-8977-2-8 (2005).
22. Secondo, L. E., Liu, N. J. & Lewinski, N. A. Methodological considerations when conducting in vitro, air–liquid interface exposures to engineered nanoparticle aerosols. *Critical Reviews in Toxicology*. , 1–32, doi:10.1080/10408444.2016.1223015 (2016).
23. Sayes, C. M., Reed, K. L. & Warheit, D. B. Assessing toxicology of fine and nanoparticles: Comparing in vitro measurements to in vivo pulmonary toxicity profiles. *Toxicological Sciences*. **97** (1), 163–180, doi:10.1093/toxsci/kfm018 (2007).
24. Maier, K. L. *et al.* Health effects of ambient particulate matter--biological mechanisms and inflammatory responses to in vitro and in vivo particle exposures. *Inhalation Toxicology*. **20** (May 2007), 319–337, doi:10.1080/08958370701866313 (2008).
25. Cohen, J. M., Teeguarden, J. G. & Demokritou, P. An integrated approach for the in vitro dosimetry of engineered nanomaterials. *Particle and Fibre Toxicology*. **11** (1), 20, doi:10.1186/1743-8977-11-20 (2014).
26. Deloid, G. *et al.* Estimating the effective density of engineered nanomaterials for in vitro dosimetry. *Nature Communications*. **5** , 3514, doi:10.1038/ncomms4514 (2014).
27. Pal, A. K., Bello, D., Cohen, J. & Demokritou, P. Implications of in vitro dosimetry on toxicological ranking of low aspect ratio engineered nanomaterials. *Nanotoxicology*. **00** (00), 1–15, doi:10.3109/17435390.2014.986670 (2015).
28. Walkey, C. *et al.* Protein corona fingerprinting predicts the cellular interaction of gold and silver nanoparticles. *ACS Nano*. **8** (3), 2439–2455, doi:10.1021/nn406018 (2014).
29. Raemy, D. O. *et al.* Effects of flame made zinc oxide particles in human lung cells - a comparison of aerosol and suspension exposures. *Particle and Fibre Toxicology*. **9** (1), 33, doi:10.1186/1743-8977-9-33 (2012).
30. Holder, A. L., Lucas, D., Goth-goldstein, R. & Koshland, C. P. Cellular response to diesel exhaust particles strongly depends on the exposure method. *Toxicological Sciences*. **103** (1), 108–115, doi:10.1093/toxsci/kfn014 (2008).
31. Sanderson, P. *et al.* Characterisation of iron-rich atmospheric submicrometre particles in the roadside environment. *Atmospheric Environment*. **140** , 167–175, doi:10.1016/j.atmosenv.2016.05.040 (2016).
32. Bartscher, H. Physical characterization of particulate emissions from diesel engines: A review. *Journal of Aerosol Science*. **36** (7), 896–932, doi:10.1016/j.jaerosci.2004.12.001 (2005).
33. Ris, C. U.S. EPA health assessment for diesel engine exhaust: a review. *Inhalation Toxicology*. **19** (Suppl. 1), 229–239, doi:10.1080/08958370701497960 (2007).
34. Jie, Y., Isa, Z. M., Jie, X., Ju, Z. L. & Ismail, N. H. *Urban vs. Rural Factors That Affect Adult Asthma. Reviews of Environmental Contamination and Toxicology*. **226** , doi:10.1007/978-1-4419-8011-3 (2013).
35. Wiemann, M., Vennemann, A., Sauer, U. G., Wiench, K., Ma-Hock, L. & Landsiedel, R. An in vitro alveolar macrophage assay for predicting the short-term inhalation toxicity of nanomaterials. *Journal of Nanobiotechnology*. **14** (1), 16, doi:10.1186/s12951-016-0164-2 (2016).
36. Kenny, L. C. *et al.* A collaborative european study of personal inhalable aerosol sampler performance. *Annals of Occupational Hygiene*. **41** (2), 135–153, doi:10.1016/S0003-4878(96)00034-8 (1997).

37. Tiwari, A. J., Fields, C. G. & Marr, L. C. A Cost-Effective Method of Aerosolizing Dry Powdered Nanoparticles. *Aerosol Science and Technology*. **47** (11), 1267–1275, doi:10.1080/02786826.2013.834292 (2013).
38. Blank, F., Rothen-Rutishauser, B. M., Schurch, S. & Gehr, P. An Optimized In Vitro Model of the Respiratory Tract Wall to Study Particle Cell Interactions. *Journal of Aerosol Medicine*. **19** (3), 392–405 (2006).
39. Laboratory, N. C. NCL Method GTA-2 HEP G2 Hepatocarcinoma Cytotoxicity Assay. (November), 1–9 (2015).
40. Kim, J. S., Peters, T. M., O'Shaughnessy, P. T., Adamcakova-Dodd, A. & Thorne, P. S. Validation of an in vitro exposure system for toxicity assessment of air-delivered nanomaterials. *Toxicology in Vitro*. **27** (1), 164–173, doi:10.1016/j.tiv.2012.08.030 (2013).
41. Mertes, P. *et al.* A compact and portable deposition chamber to study nanoparticles in air-exposed tissue. *Journal of aerosol medicine and pulmonary drug delivery*. **26** (0), 228–35, doi:10.1089/jamp.2012.0985 (2013).
42. Panas, A. *et al.* Silica nanoparticles are less toxic to human lung cells when deposited at the air–liquid interface compared to conventional submerged exposure. *Beilstein Journal of Nanotechnology*. **5**, 1590–1602, doi:10.3762/bjnano.5.171 (2014).
43. Zavala, J. *et al.* Regulating temperature and relative humidity in air–liquid interface in vitro systems eliminates cytotoxicity resulting from control air exposures. *Toxicology Research*. **6**, 448–459, doi:10.1039/C7TX00109F (2017).
44. Jing, X., Park, J. H., Peters, T. M. & Thorne, P. S. Toxicity of copper oxide nanoparticles in lung epithelial cells exposed at the air – liquid interface compared with in vivo assessment. *TOXICOLOGY IN VITRO*. **29** (3), 502–511, doi:10.1016/j.tiv.2014.12.023 (2015).
45. Bitterle, E. *et al.* Dose-controlled exposure of A549 epithelial cells at the air-liquid interface to airborne ultrafine carbonaceous particles. *Chemosphere*. **65**, 1784–1790, doi:10.1016/j.chemosphere.2006.04.035 (2006).
46. Steinritz, D. *et al.* Use of the Cultex Radial Flow System as an in vitro exposure method to assess acute pulmonary toxicity of fine dusts and nanoparticles with special focus on the intra- and inter-laboratory reproducibility. *Chemico-Biological Interactions*. **206** (3), 479–490, doi:10.1016/j.cbi.2013.05.001 (2013).
47. Cronholm, P. *et al.* Intracellular uptake and toxicity of Ag and CuO nanoparticles: A comparison between nanoparticles and their corresponding metal ions. *Small*. **9** (7), 970–982, doi:10.1002/sml.201201069 (2013).
48. Cronholm, P., Midander, K., Karlsson, H. L., Elihn, K., Wallinder, I. O. & Möller, L. Effect of sonication and serum proteins on copper release from copper nanoparticles and the toxicity towards lung epithelial cells. *Nanotoxicology*. **5** (2), 269–281, doi:10.3109/17435390.2010.536268 (2011).
49. Midander, K. *et al.* Surface characteristics, copper release, and toxicity of nano- and micrometer-sized copper and copper(II) oxide particles: A cross-disciplinary study. *Small*. **5** (3), 389–399, doi:10.1002/sml.200801220 (2009).



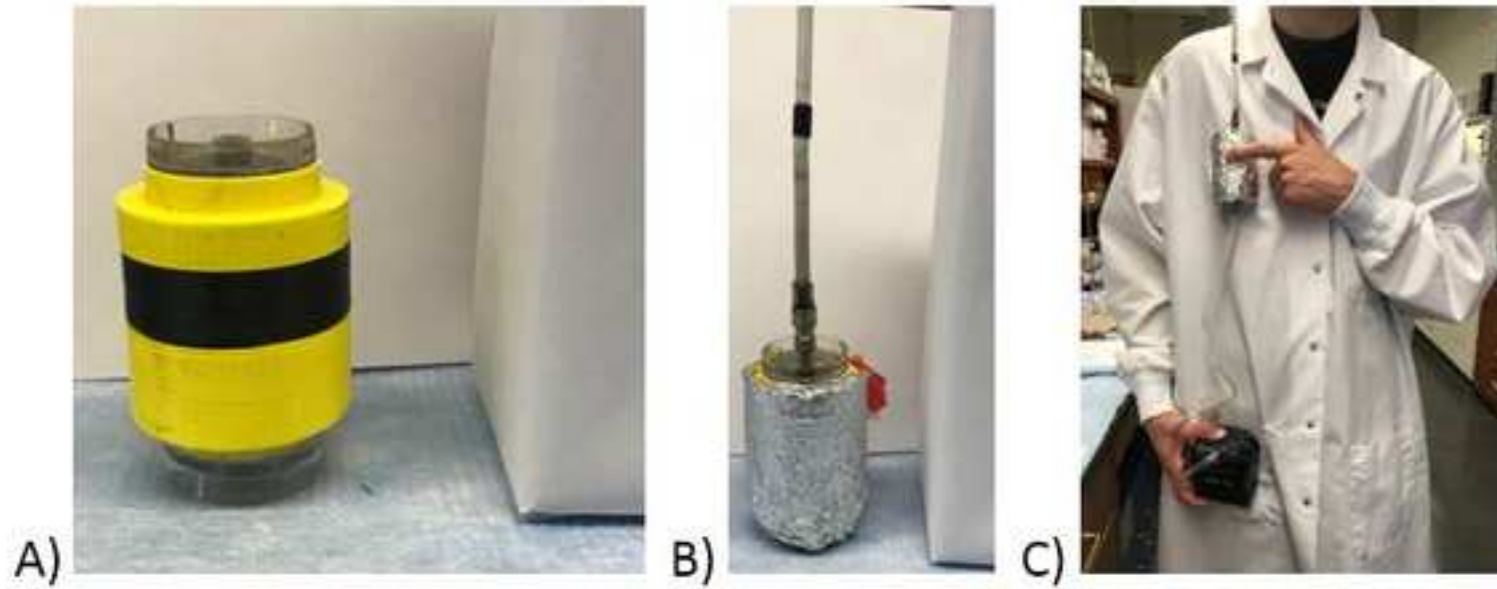


Figure 3

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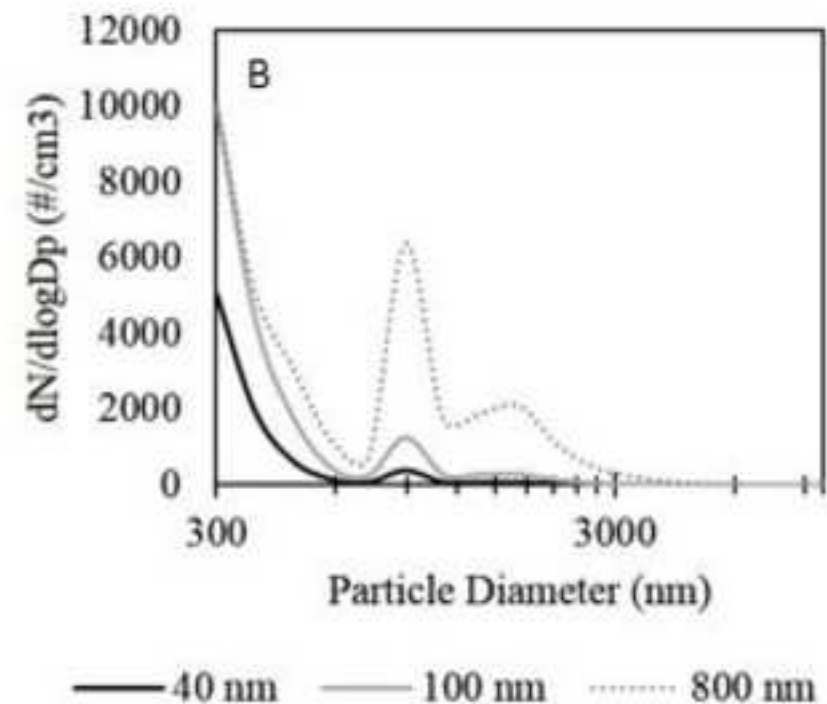
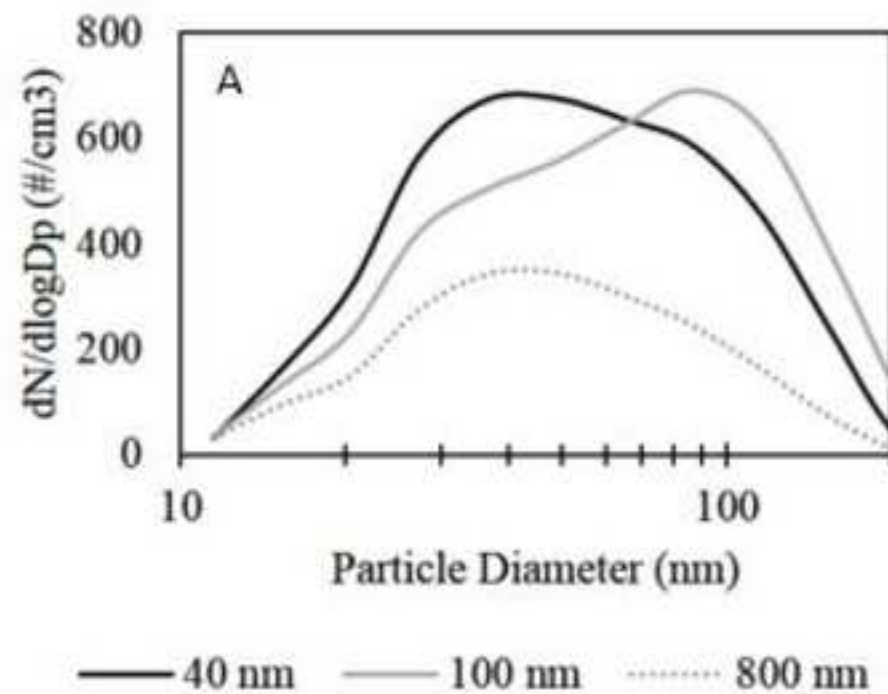


Figure 4

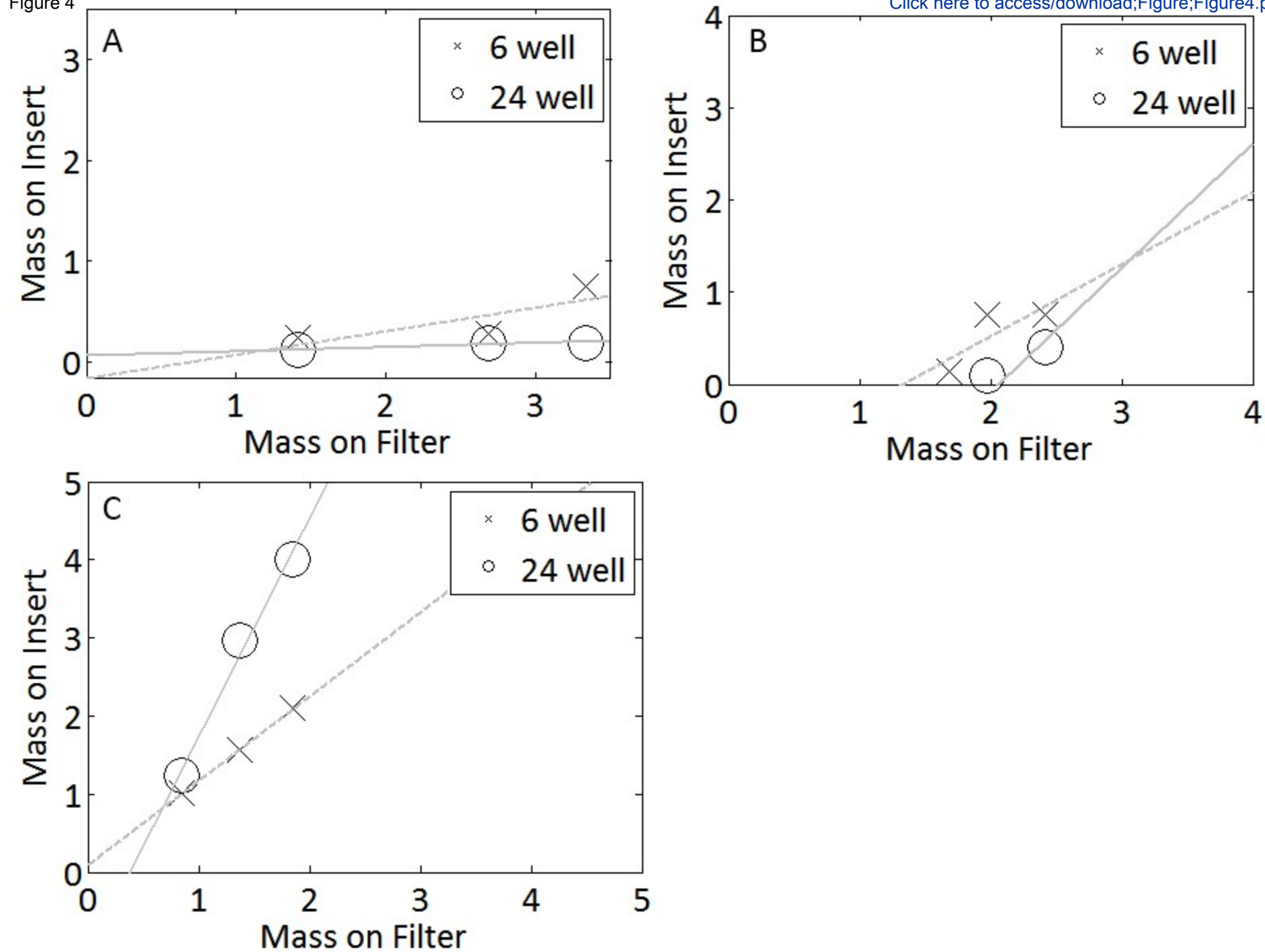
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Figure 5

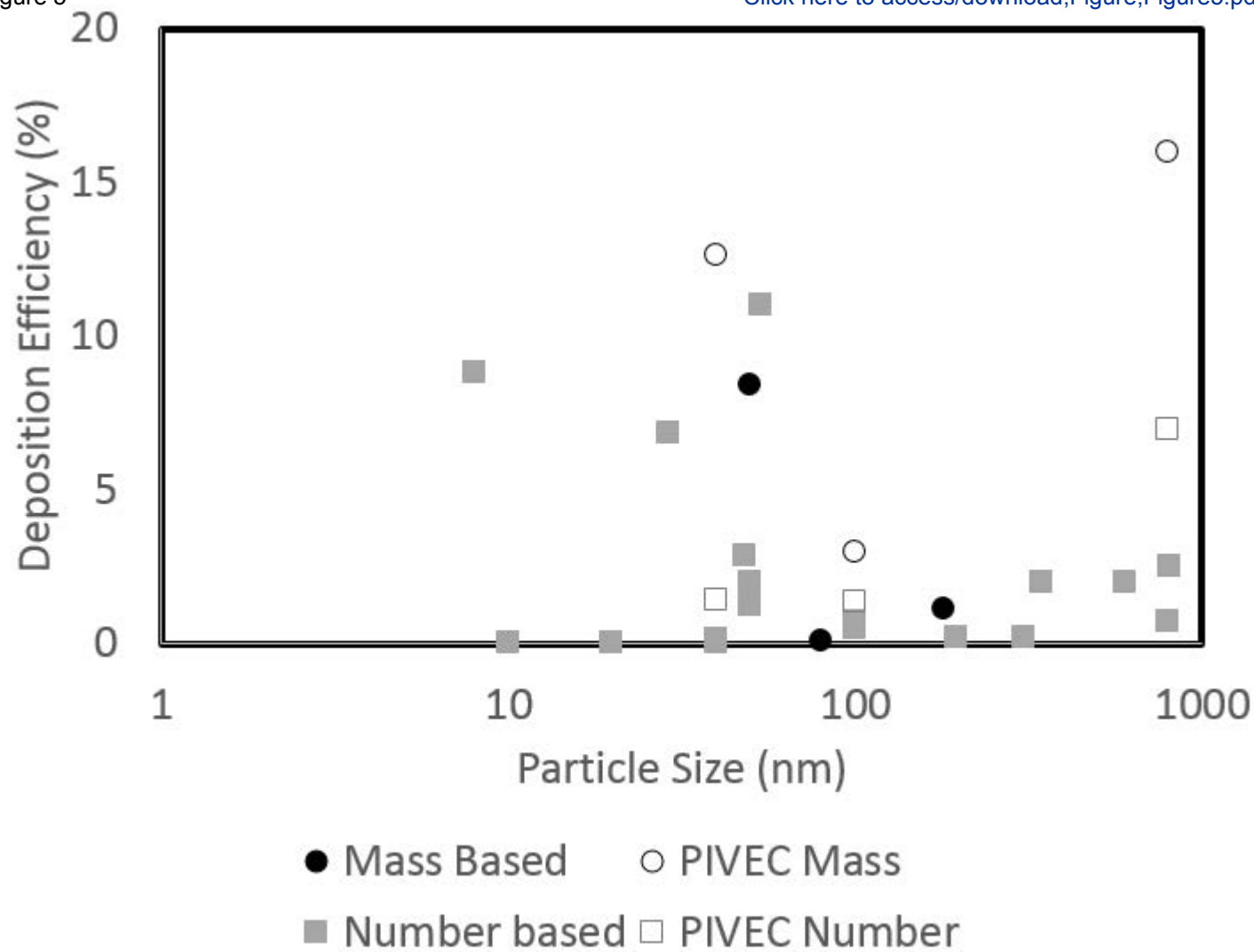
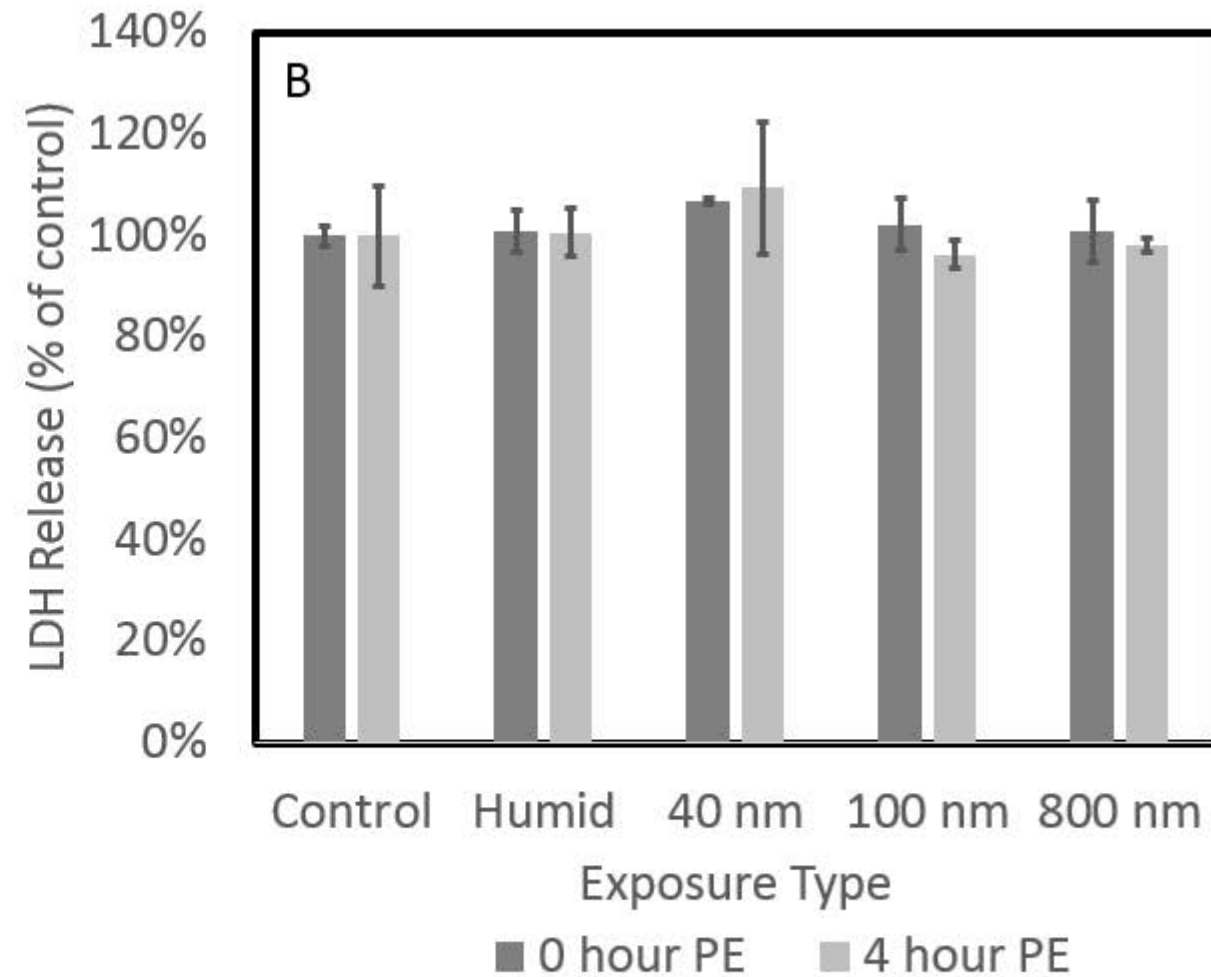
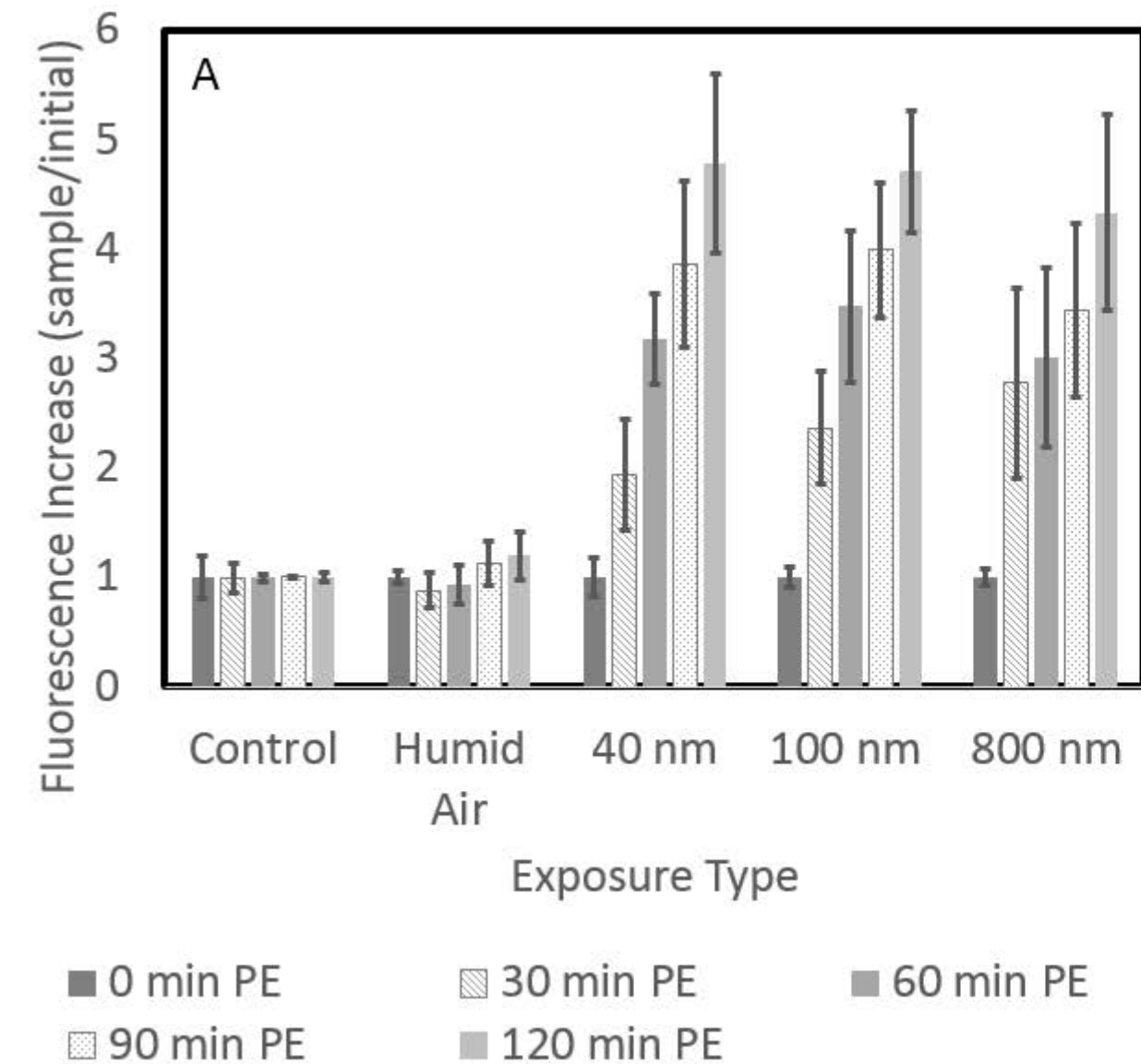
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Figure 6

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		Number Based Deposition Efficiency (%)			Mass Based Deposition Efficiency (%)		
6 well	40 nm	17.83	±	32.13	5.85	±	0.85
	100 nm	0.47	±	4.06	5.11	±	0.94
	800 nm	3.70	±	35.00	6.39	±	1.01
24 well	40 nm	1.43	±	2.43	12.61	±	1.34
	100 nm	1.37	±	19.45	2.95	±	0.75
	800 nm	6.98	±	3.93	15.95	±	0.53

Name of Material/ Equipment	Company	Catalog Number
Scanning mobility particle sizer (SMPS)	TSI, Inc.	3910
Optical particle sizer (OPS)	TSI, Inc.	3330
Stainless Steel Pipe, 4" Long	McMaster-Carr	4830K116
Brass Ball Valve with Lever Handle	McMaster-Carr	4112T12
Steel Pipe, 2" Long	McMaster-Carr	7753K121
HEPA filter	GE Healthcare	09-744-12
Vacuum Generator	PISCO USA	VCH10-018C
PIVEC	VCU	
Resistive heater		
1/4" barbed connectors	Zefon International, Inc.	459743
Porous tubing	Scientific Commodities, Inc.	BB2062-1814A
Battery power bank		
Cell culture insert	Fisherbrand	353095
Filter Forceps	Fisherbrand	09-753-50
Transfer Pipette	ThermoScientific	13-711-27
Glass Fiber Filters	SKC	225-7
Ultra Micro Balance	A&D	BM-22
37 mm filter cassette	SKC	225-3250
Variable flow vacuum pump	SKC	220-5000TC
Copper Particles	U.S. Research Materials, Inc.	US1090
Copper Particles	U.S. Research Materials, Inc.	US1088
Copper Particles	U.S. Research Materials, Inc.	US1117M

Comments/Description
NanoSMPS
Standard-Wall 304/304L, Threaded on Both Ends, 1/8 Pipe Size
Compact High-Pressure Rating, 1/8 NPT Female
Standard Wall, Threaded on One End, 1/8 Pipe Size
HEPA-Cap Disposable Air Filtration Capsule
For design please contact authors
Hydrophilic 10 um pores
24 well plate insert
Binder-Free Type AE Filter 37 MM 1.00 um pore
Housed in environmental chamber
Filter Cassette Blank, 37 mm, Clear Styrene
AirChek TOUCH, 5 to 5000 mL/min
40 nm
100 nm
800 nm



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Article Title:	A New Portable In Vitro Exposure Cassette for Aerosol Sampling		
Signature:			Date: August 6, 2018

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Dear Dr. Steindel:

Enclosed please find our responses to the reviewers' comments and the revised manuscript. We would like to thank the reviewers for their thoughtful critiques of the manuscript. The comments and recommendations of the reviewers have helped us improve the presentation and clarity of the method. Please find below response to the individual reviews. Changes to the manuscript were incorporated in red font. We hope that our specific responses to the reviewers adequately address any concerns. Thank you for considering the revised manuscript.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been thoroughly proofread, ensuring there are no spelling or grammatical issues.

2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to ..."

The summary has been rephrased to clearly describe the protocol and applications. "Here, we present a protocol to perform portable cellular aerosol exposures and measure cellular response. The method uses cells grown at the air-liquid interface, mimicking *in vivo* physiology. Cellular response to copper nanoparticles was observed as oxidative stress through reactive oxygen species generation and cytotoxicity as lactate dehydrogenase release."

3. Please rephrase the Abstract to more clearly state the goal of the protocol.

The abstract has been re-written to more clearly state the goal of the protocol. "This protocol introduces a new *in vitro* exposure system, capable of being worn, including its characterization and performance. Air-liquid interface (ALI) *in vitro* exposure systems are often large and bulky, making transport to the field and operation at the source of emission or within the breathing zone difficult. Through miniaturization of these systems, the lab can be brought to the field, expediting processing time and providing a more appropriate exposure method that does not alter the aerosol prior to contacting the cells. The Portable *In Vitro* Exposure Cassette (PIVEC) adapts a 37 mm filter cassette to allow for *in vitro* toxicity testing outside of a traditional laboratory setting. The PIVEC was characterized using three sizes of copper nanoparticles to determine deposition efficiency based on gravimetric and particle number concentration analysis. Initial cytotoxicity experiments were performed with exposed lung cells to determine the ability of the system to deposit particles while maintaining cell viability. The PIVEC provides a similar or increased deposition efficiency when comparing to available perpendicular flow *in vitro* exposure devices. Despite the lower sample throughput, the small size gives some advantages to current *in vitro* ALI exposure systems. These include the ability to be worn for personal monitoring, mobility from the laboratory to the source of emission, and the option to set-up multiple systems for spatial resolution while maintaining a lower user cost. The PIVEC is a system capable of collecting aerosols in the field and within the breathing zone onto an air-interfaced, *in vitro* model."

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

1.1.1: Please specify the size of the tubing and connectors.

1.1.1 Make sure to use new or well cleaned **with 70% ethanol ¼” inner diameter** tubing and **¼” outer diameter** connectors for system assembly.

1.1.2: Please specify the temperature and humidity suggested for storing the test materials. A schematic of the system and setup as Figure 1 would greatly aid in step 2.1 of the protocol.

1.1.2 Store test materials **including filters, PIVEC components, tweezers, and particle powders** in a well-controlled environment, with respect to temperature and humidity, for at least 24 hours prior to experiment. **The temperature should be near room temperature, approximately 20 C, with relative humidity less than 35%.** This is very important to achieve repeatability between experiments.

3.1.1: Please specify the pore size of the filters used.

3.1.1 Keep **1.00 µm pore glass fiber filters** under low humidity conditions for at least 24 hours prior to pre-exposure measurements. Weigh the filter three times and record the filter weights. Place the filter in a cell culture insert.

4.1.2: How to obtain the mass-based deposited dose?

4.1.2 Use administered mass, m_{admin} , and mass-based deposited dose **determined in 4.1.1**, m_{dep} , to calculate the mass-based deposition efficiency, η_m , for experiment.

5. Please include single-line spaces between all paragraphs, headings, steps, etc.

The manuscript has been revised to include the single-line spaces throughout.

6. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Approximately two pages of the protocol has been highlighted in yellow. This covers steps 2 and 5 to show the set-up of the system and exposure of cells to a dry aerosol.

7. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Steps 2 and 5 were highlighted. This will show the set-up of the aerosol and PIVEC exposure system and exposure of cells within the PIVEC to a dry aerosol.

8. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

All steps within the sections have been highlighted.

9. Please number the figures in the sequence in which you refer to them in the manuscript text. Currently Figure 2 (line 164) is mentioned before Figure 1.

Thank you for bringing this to our attention. The figures have been renumbered and figure captions adjusted accordingly. Line 164, Lines 292-296.

10. Lines 310-311: Please remove commercial language including trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. For example: Cultex CG, Vitrocell.

The commercial language have been removed from the manuscript.

From the Introduction: “Perpendicular flow, used by a **microsprayer**,⁹ **MINUCCELL**,¹² **NACIVT**,¹³ and **commercial ALI systems**^{10,11,14}, adds the impaction of particles within the deposition region.”

From the Representative Results: “Compared to similar systems throughout literature, including the **MINUCCELL**^{14,60} and **NACIVT**⁶¹ systems, the deposition efficiency of the PIVEC over the range of particle sizes tested is comparable or increased over reported values, observed in Figure 4.”

11. Discussion: Please describe critical steps within the protocol.

The discussion of critical steps within the protocol has been more explicitly revisited in the Discussion section from the Results section. “Deposition can be increased by changing the flow rate and exposure duration. This may also influence the cell viability with the potential of drying out the cells. The conditioning of aerosols is often performed to compensate for physiological attributes such as body temperature and humidification in the airways. Increased humidity, over 50%, mimics inhaled air and decreases cell death due to vehicle exposure.⁶³ When temperature and humidity are not well controlled, the cellular response can be influenced. By decreasing the flow rate, additional particles of all sizes will deposit, increasing the deposition. Exposure duration is proportional to deposition, allowing more particles to deposit over an extended experimental period. Conditioning of the aerosol is important when increasing the exposure duration so that the cells do not dry out which can affect biological responses. **Additionally, by conditioning the filters as described in Step 1 of the protocol, the deposition efficiency can be accurately determined.**”

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have described a portable in vitro exposure sampling method.

Major Concerns:

None

Minor Concerns:

Even the system is considered small and portable this system still requires SMPS/OPS which are considerably large instruments for the setup.

Revisions needed in introduction, methods, results, discussion, and references sections. See below

Introduction:

Authors Introduced the subject well. . The authors describe the disadvantages of other ALI systems due to their size, how it affects the data (line 97..). Including more details how the PIVEC system overcomes the described issues can be more meaningful to the reader. The authors can include some further information comparing the PIVEC to other ALI systems comparing not only particle exposure but also gaseous exposures and measurements. Visualizing the PIVEC system is difficult without mentioning the dimensions and weight, including these may help the reader.

We thank the reviewer for their insight. Additional information has been added to the Introduction section discussing the ability of the PIVEC to overcome described issues, including the dimensions and weight of the system. Additional comparisons between the PIVEC and other ALI systems is found in the Results and Discussion sections. **“The PIVEC is smaller than current systems, with an external surface area of approximately 460 cm² and weighing only 60 grams, with thermal and humidity control incorporated into the system allowing for a highly portable device. The decreased size and weight allows the system to be worn or taken to the source of exposure, permitting direct sampling.”**

Protocol:

Well written. Figure 1 is not very clear. Increasing the clarity of the pictures and labeling the pictures arrows may be helpful to the reader. For 6 and 24 well plates, can cells in actual ALI conditions be used? If so how are the ALI conditions achieved in this system? Include some details (line 198).

Thank you for helping us clarify this. For both 6 and 24 well plates, cells in the ALI conditions can be used. To aid the reader in understanding how ALI conditions are achieved, an additional section, 5.1 Culture Cells at Air-Liquid Interface, has been added to the protocol.

“5.1 Culture Cells at Air-Liquid Interface

5.1.1 Lift cells from culture flask by adding trypsin-EDTA, 3 mL for a T75 flask or 1 mL for a T25 flask, and incubate for 5 minutes at 37°C. Add complete media to flask and rinse flask wall with cell suspension to maximize the recovered cell number. Transfer cell suspension to a sterile 15 mL conical tube then centrifuge cells at 1200 rpm for 3 minutes.

5.1.2 Remove supernatant containing trypsin-EDTA and resuspend cell pellet in 10 mL of complete media. Remove 10 µL of cell suspension and add to hemocytometer. Count cells in hemocytometer to determine the concentration and total number of cells.

5.1.3 Add complete media to each well in well plate, for 24 well plate add 0.5 mL to each well. Place cell culture inserts in wells. Seed cell culture inserts on apical side at a cell density near 1×10^5 cells/cm² for cell types that grow at a rate near doubling per day. Note: Cells with a slower growth rate can be seeded at an increased cell density.

5.1.4 Add complete media to apical side of transwell to reach final volume, for 24 well plate final volume is 0.25 mL.

5.1.5 Culture for 7 days in submerged conditions, replacing media every 1-2 days. After 7 days, remove apical media and culture for at least 1 day in ALI conditions, replacing only the basolateral media.”

Results:

The deposition efficiencies are still very low. Can this be improved? How uniform is the deposition between wells (6 or 24)? Are there any data to show equal deposition between the wells?

The reviewer is correct that the deposition efficiencies are still low for an ALI exposure system. The deposition efficiency can be improved within the system and will change for different aerosol compounds. The issue of uniform deposition is also addressed within the revised manuscript. “The deposition in the 24 wells is very uniform over the insert, however, deposition in the 6 well design is lacking uniformity as most of the particles deposit near the center of the insert.” ... “The deposition efficiency within the PIVEC can be improved through minimizing losses to the system using electrostatic dissipative or conductive plastic or similar material to design the PIVEC.”

Figure 3: Clearly label a, b, and c.

The figures are labeled in the upper left corner of the chart.

Figures 5 and 6: Show statistical comparisons.

Figure legends: Include statistical comparisons and p values for the figures.

Thank you for helping us to clarify the statistical comparisons of these measurements. The manuscript has been adjusted to reflect these changes. “Figure 5. Cellular Response to Copper Nanoparticles Post-Exposure (PE). For all measurements, n=3 and p<0.05. A) Oxidative Stress determined using the DCFH-DA Assay. B) Cytotoxicity determined using the LDH Assay.”

Include a section on statistical methods.

Thank you for the suggestion. A section on statistical methods used has been included in the updated protocol.

“6 Statistical Methods

6.1 Analyzation of Biological Assay Data

6.1.1 Report ROS production as the fluorescence intensity increase of treated cells relative to baseline measurements. Report LDH activity as the fluorescence intensity increase of treated cells relative to untreated cells.

6.1.2 Perform single factor ANOVA to determine statistical differences between data sets. Where appropriate, perform student t-tests at a value of significance of 0.05. Report data as the mean \pm standard deviation of at least three exposure measurements.”

Discussion section: The discussion section very short. Consider expanding based on the results.

The discussion section has been expanded with relationships of the deposition and exposures within the PIVEC to alternative perpendicular flow systems.

References: Too many references, maybe reduce by including only the most relevant references.

The number of references cited has been reduced to 49 from 65.

Reviewer #2:

Manuscript Summary:

The authors describe a portable system to expose cells at the air-liquid interface to detect cytotoxic effects of aerosols for personal monitoring in the breathing zone. In general, this is a challenge which has been implemented.

They used three different sizes of Cu nanoparticles to generate aerosols, to determine the deposition efficiency and to expose cells.

In summary, the manuscript is well written and the well-structured figures clearly demonstrate the results.

Major Concerns:

The data of the aerosol characterization (mass concentration, number concentration) are missing.

Thank you for bringing this to our attention. The SMPS and OPS measurements for each aerosol are included now as Figure 3. "Figure 3. Particle number concentration of copper nanoparticle aerosols. A) SMPS measurement. B) OPS measurement."

Which method was used to humidify the aerosol?

The aerosol was humidified using a porous, wetted tube. This is now reflected in the manuscript. "The temperature was maintained through the use of a battery-powered resistive heater and the aerosol humidified using increased natural humidification through a porous, wetted tube."

Page 9, line 320: the flow rate over the cells is 0.5 LPM. This flow seems extremely high for the small inserts (24-well plate inserts) in comparison to the flows used in other studies using CULTEX, MINUCCELL, NACIVT or VITROCELL systems, which use flows between 0.005 and 0.1 LPM for bigger inserts (6-well plate inserts). Such a high flow rate would probably damage the cells by shear stress.

The reviewer brings up a valid point that the increased flow rate may damage the cells via shear stress. To address this, the following line has been added to the manuscript "Alternative perpendicular flow exposure systems use between 0.005 LPM and 1.5 LPM for a sustained exposure period whereas this method uses a moderate flow rate during a rapid exposure."

Minor Concerns:

Which types of cells were used to create the data in Fig. 5?

Thank you for bringing this to our attention. The cells used in this study was the A549 cell line, an alveolar epithelial cancer cell commonly used in inhalation toxicology. "A549 cells, an alveolar epithelial carcinoma cell line, were exposed for 10 minutes to varying sizes of copper nanoparticles at a flow rate of 0.5 LPM."

Page 3, line 79: It should be specified that only primary lung cells are able to generate a differentiated cell monolayer.

The reviewer is correct that only primary lung cells will generate a differentiated cell monolayer. Cell lines, however, will produce a polarized barrier that may reflect some *in vivo* characteristics depending on the line and method of culture. "Lung cells grown at the ALI have the ability to generate a polarized barrier layer,²¹⁻²⁴ which produces physiological traits that resemble the *in vivo* lung epithelium, including mucus and surfactant production in specific bronchial or alveolar cell lines, cilia beating,²² tight junctions,^{22,25} and cell polarization.²¹"

Page 5, line134: "... using *in vitro* exposures with confirmation *in vitro*." I guess that one of the terms "*in Vitro*" should be "*in vivo*"

Thank you for pointing this out. The reviewer is correct and the appropriate change has been made. "Out of ten nanosized metal oxides tested, six tested as active (titanium oxide, zinc oxide, and

four different cerium oxide) using exposures *in vitro* with confirmation *in vivo*.”

Only few tube materials are suitable for conducting aerosols. Therefore, the material of the tubings should be known.

The reviewer is correct that only few tube materials are suitable for conducting aerosol experiments due to potential interactions and losses to the tube. In this study, conductive tubing was used to decrease these losses.

Page 9, line 323: "Cytotoxicity and oxidative stress were observed within the first twenty-four hours of exposure." This is misleading. In Line 319 it is written that the exposure was for 10 min and here for 24h?

Thank you for bringing this to our attention. The exposure was for 10 minutes and biological endpoints were observed within 24 hours post-exposure. The manuscript has been amended to make this clearer. "Cytotoxicity and oxidative stress were observed within the first twenty-four hours post-exposure."

Page 9, line 325: In the text it is said that figure 5b shows results of "4h and 24h post-exposure", however figure 5 does not show the 24h results. Later on it is said that the 24h viability results are not shown, why not? This would be interesting for the reader. This could also show if exposure of cells themselves was cytotoxic or the exposure to the nanoparticles.

Thank you for helping us to clarify this in the text. The 24 hour viability results were not shown as there was significant variability between experiments. However, within this study, it does appear as though the exposure itself produced some cytotoxicity.

"Cytotoxicity was measured using the release of lactate dehydrogenase (LDH) from damaged cells immediately, 4 hours, and 24 hours post-exposure. There was no significant toxicity from copper nanoparticles below 1.62 mg/cm² within 4 hours of exposure, Figure 6b."

Figure 5: please explain the abbreviation "PE" in the legend.

The manuscript has been adjusted to reflect this addition. "Figure 6. Cellular Response to Copper Nanoparticles Post-Exposure (PE). A) Oxidative Stress determined using the DCFH-DA Assay. B) Cytotoxicity determined using the LDH Assay."