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# Cellular Response to Diesel Exhaust Particles Strongly Depends on the Exposure Method

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***In vitro* exposure to aerosols at the air–liquid interface (ALI) preserves the physical and chemical characteristics of aerosol particles. Although frequently described as being a more physiologic exposure method, ALI exposure has not been directly compared with conventional *in vitro* exposures where the particles are suspended in medium. We exposed immortalized human bronchial epithelial cells (16HBE14o) to aerosolized diesel exhaust particles at the ALI and to suspensions of collected particles. The response of the cells was determined from measurements of the cell viability and interleukin-8 (IL-8) secretion. The deposited size distribution at the cell surface was measured with transmission electron microscopy to obtain a dose for the ALI exposure. Although exposure by either method caused a slight decrease in cell viability and induced IL-8 secretion, the response to ALI exposure occurred at doses several orders of magnitude lower than exposure to particles in suspension. The most likely sources for the different dose responses are the artifacts introduced during the collection and resuspension of particles for conventional suspension exposures. The number concentration of particles deposited at the ALI is similar to the modeled deposition in the tracheal–bronchial region in a human lung, but the ALI size distribution is skewed toward particles larger than those deposited in the lung.**

***Key Words:* air–liquid interface; particle deposition; ultrafine particles; nanoparticle toxicology.**

The toxicity of an aerosol is dependent on the particle's physical and chemical characteristics (Maynard and Kuempel, 2005). Particles are frequently present in a mixture with gas phase and semivolatile species that can be present both as a gas and adsorbed to the particle, which may affect the resulting toxicity. A multitude of aerosols with a variety of characteristics exist in environmental and occupational settings, making it important to develop rapid assays that can be used to assess toxicity and predict associated human health effects.

*In vitro* screening assays are desirable because they can be used to test a number of parameters for many different particle types. However, conventional *in vitro* assays cannot accurately assess the toxicity of aerosols such as diesel exhaust, which are in equilibrium with a variety of associated semivolatile compounds. Conventional *in vitro* assays are limited by the requirement that cells are exposed to particles collected and resuspended in the cell growth medium. Semivolatile compounds can be lost in the collection process and the suspension process is likely to change the surface chemistry, morphology, and size of particles. *In vitro* exposure at the air–liquid interface (ALI) avoids some of these artifacts by exposing cells directly to the aerosol. This approach has been used to investigate the effects of a variety of different particles important for human health such as cigarette smoke (Aufderheide and Mohr, 2004; Aufderheide *et al*., 2003; Olivera *et al*., 2007), diesel exhaust (Seagrave *et al*., 2007), gasoline exhaust (Cheng *et al*., 2003), other combustion-generated particles (Diabate *et al*., 2002), and metal nanoparticles (Cheng, 2004). The ALI exposure has also been used to assess the toxicity of a variety of gas phase species such as nitrogen dioxide (Ritter *et al*., 2001), ozone (Ritter *et al*., 2001), simulated smog (Doyle *et al*., 2004), and volatile organic compounds (Bakand *et al*., 2006). The objective of this study is to compare ALI with conventional *in vitro* exposure to diesel exhaust particles, a ubiquitous environmental pollutant that has been extensively investigated with conventional *in vitro* methods.

One of the challenges faced with *in vitro* exposure studies is determining dose. Without a dose measurement, the results from an *in vitro* exposure cannot be directly compared with other exposure methods such as animal inhalation or instillation. Furthermore, it is difficult to relate results to relevant human exposures. In conventional *in vitro* exposures, the dose to which cells are exposed is different than that in the culture medium, making the actual exposure-dose difficult to determine (Teeguarden *et al*., 2007). In ALI experiments, dose is commonly represented as dilution ratio and exposure duration, along with measurements of aerosol mass or number concentration. The actual amount deposited on the cell layer is dependent upon flow conditions and particle properties, which vary by ALI exposure configuration and particle type.

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At high aerosol mass concentrations (133–840 mg/m$^3$), gravimetric measurement of the deposited dose can be performed (Massey *et al.*, 1998). However, many particle exposures occur at much lower concentrations. For example, the United States twenty-four-hour ambient 2.5-μm particulate matter standard is 35 μg/m$^3$ (Federal Register, 2006); at this concentration the deposited mass is too small to detect gravimetrically. To address this, fluorescent test aerosols have been used to measure smaller amounts of particle deposition. Deposition rates have been determined from the fluorescence of extracts of wells exposed to either monodisperse beads (140 and 500 nm) (Seagrave *et al.*, 2007) or polydisperse fluorescein aerosols (Mulhopt *et al.*, 2004). Mass deposition of tobacco smoke at the ALI has also been measured from the fluorescence of well extracts (Aufderheide *et al.*, 2003).

In the previously described dose measurements, deposition was measured only in terms of mass, which is not sufficient to determine the effect of particle size or number on their toxicity. One approach to obtain the size and number deposited is through the development of analytic models. Tippe *et al.* (2002) developed a simple model to predict the particle deposition at the ALI for particles with diameters ranging from 75 to 1000 nm. They used an impaction type ALI exposure, where the aerosol flow is directed perpendicular to the cell layer. The model was validated with counts of deposited monodisperse particles made from scanning electron micrographs. A more detailed computational model was developed by Desantes *et al.* (2006), which estimated the deposition efficiency for several flow rates, particle densities, and diameters (10–1000 nm). However, to our knowledge there are no measurements of a deposited size distribution for an ALI exposure, therefore this is one of the objectives of this study. With a known deposited size distribution, we can compare the dose–response relationship of the ALI and suspension exposure methods. Furthermore, we can compare the deposition at the ALI to that typical for a human lung.

## MATERIALS AND METHODS

We previously exposed a human bronchial epithelial cell line to diesel exhaust particles by ALI exposure and investigated the effects of the exhaust gases by filtering (Holder *et al.*, 2007). We draw on those results for the whole and filtered exhaust ALI exposure used here for comparison with diesel exhaust particle suspensions exposures. Some details of the ALI exposure are repeated for clarity; a comprehensive description of the ALI exposure methodology is in Holder *et al.* (2007).

***Cell cultures.*** The immortalized human bronchial epithelial cell line, 16HBE14o (gift from Dieter Gruenert, Pacific Medical Research Center) was used for all exposures (Cozens *et al.*, 1994). Cells were maintained in the logarithmic phase of growth in collagen-coated flasks in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, glutamine, streptomycin (100 μg/ml), and penicillin (100 U/ml), and were maintained under humidified conditions in a 5% $CO_2$ atmosphere at 37°C.

For exposure to the particle suspension, cells were seeded onto 35-mm collagen-coated cell culture dishes at $1.0 \times 10^5$ cm$^{-2}$. For ALI exposure, 6- and 24-well plate collagen-coated Transwells (Corning, Acton, MA), with growth areas of 4.7 and 0.33 cm$^2$, respectively, were seeded with cells at $1.0 \times 10^5$ cm$^{-2}$ and grown submerged for two days to achieve confluence. After ALI and suspension exposures, cell viability was measured with an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide kit (Sigma, St. Louis MO) and interleukin-8 (IL-8) secretion was measured with an enzyme linked immunosorbent assay (ELISA) kit (Invitrogen, Carlsbad, CA) (Holder *et al.*, 2007).

***Particle source.*** The particle source for the exposures was described in detail in Holder *et al.* (2007). Briefly, a single cylinder diesel engine (Acme Motori, Treviso, Italy Model ADX-300, Treviso, Italy) was used as a source of aerosol particles. The engine was operated on a commercial certified California #2 diesel fuel (approximately 400 ppm sulfur). Exhaust was diluted and aged in a chamber ($2.2 \times 2.4 \times 4.6$ m$^3$) with a 20-min residence time. During the ALI exposure, the average chamber temperature and relative humidity were 20°C and 45%, and the average pollutant concentrations were 7 ppm for $NO_X$, and 0.1% for $CO_2$.

***Particle exposure at ALI.*** In preparation for ALI exposure, the Transwell growth medium was replaced with Laboratory of Human Carcinogenesis (LHC) medium containing 2 mM HEPES buffer to ensure that the pH is unaffected by the low $CO_2$ levels during the exposure. Empty wells were filled with water to increases the humidity in the cell enclosure. A control group was left in the incubator for the duration of the exposure. The exposed Transwell plates were contained in a sealed stainless steel enclosure (7.6 cm × 14 cm × 21.6 cm) inside of an incubator kept at 37°C. A pump drew diesel exhaust into the cell enclosure at 1.3 liter per minute (lpm) (1.8-min residence time). Flow into the enclosure is laminar, and is expected to be laminar above the Transwell plate (chamber Reynolds number = 14). The cells were exposed to diesel exhaust for 6 h. A concurrent exposure was performed with filtered diesel exhaust to account for the effects of the exhaust gases. After the exposure, the medium was removed, the cells were rinsed, and MEM was added. The cells were incubated for 20 h before the medium was removed for IL-8 assay (three wells from the six-well plate) and the cell viability was measured (four wells from the 24-well plate).

***Particle exposure in suspension.*** Particles from the diesel engine were collected from the walls of the exhaust sample tubing. The tubing is oriented vertically, thus particle deposition is primarily by diffusion and thermophoresis due to the temperature differences between the hot exhaust gases and the colder tube. Both mechanisms tend to deposit smaller particles (< 1 μm) more effectively than larger particles. The particles are suspended in distilled water with 0.04% dipalmitoyl phosphatidylcholine (DPPC). The DPPC was used to disperse the diesel particles, which tend to aggregate in water. The suspension was stirred for about 2 min on a vortex stirrer to disperse the particles, and 0.2-ml aliquots were added to the cells in LHC medium to give concentrations of 0, 0.5, 1, 7.5, 10, and 50 μg/ml of particles, each with 0.004% DPPC. Each concentration was tested in triplicate wells. The cells were incubated (at 37°C and 5% $CO_2$) for 6 h. The cells were then rinsed twice with phosphate buffered saline, MEM was added, and the cells were incubated for 20 h. The medium was removed for IL-8 assay and the cell viability was measured.

***Deposition measurement.*** Measurement of the particle deposition was made using the cell enclosure and a test aerosol as shown in Figure 1. A downward flow diffusion methane–air flame was used as a stable source of combustion-generated particles for the test aerosol as described in Stipe *et al.* (2005). The methane and air-flow rates were adjusted to achieve a size distribution matching the diesel particle size distribution measured during the ALI exposure. A sample from the postflame region was dried in a silica gel diffusion dryer and drawn into the cell enclosure under the same flow conditions used in the ALI exposure. Measurements of the particle size distribution inside the cell enclosure were made with a TSI (Shoreview, MN) scanning mobility particle sizer (SMPS, model 3077 differential mobility analyzer, model 3025 condensation particle counter).

A transmission electron microscope (TEM) grid placed in a central well of a six-well plate was exposed for 6 h, matching the previous ALI exposure condition. A random portion of the grid was selected for imaging with an FEI Tecnai 12 TEM. All particles in the area were counted and measured to determine the deposited number and size distribution.

A test aerosol is used for the deposition measurement because the downward flow flame is stable (approximately 4% variation) and can be adjusted to

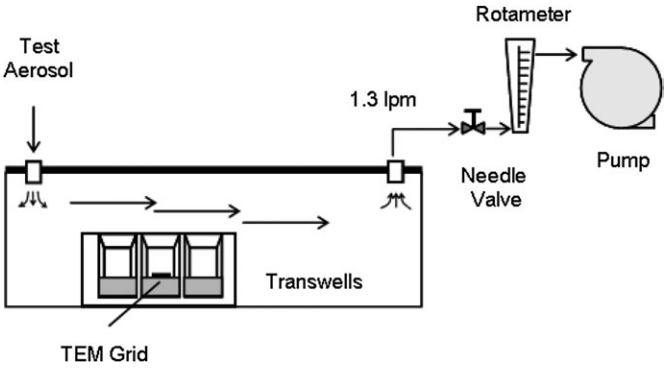


**FIG. 1.** Schematic of the cell enclosure configuration used for diesel ALI exposure and measurement of particle deposition.

produce a desired particle size and number concentration (Stipe *et al*., 2005). The downward flow flame produces particles with similar characteristics to diesel particles (Table 1). Diesel and flame generated particles both consist of aggregates of spherical primary particles with a fractal dimension ranging from 1.6 to 1.9 (Koylu *et al*., 1995; Park *et al*., 2004). The diesel and flame generated particles have approximately the same size-dependent dynamic shape factor (Slowik *et al*., 2004). However, our test aerosol has a slightly smaller primary particle diameter. Additionally, the diesel particles have a size-dependent density; smaller diesel particles have a lower density due to increased organic carbon content (Park *et al*., 2004).

The irregular shape of combustion-generated particles makes it necessary to relate the sizes measured with quantities that describe the particle's behavior. The SMPS measures a mobility diameter, and the TEM image provides a projected area diameter. For particles with a fractal dimension of approximately 1.75, the mobility diameter is roughly equivalent to the projected area diameter (Rogak *et al*., 1993). The volume equivalent diameter is the diameter of a sphere with the same volume as the particle and for irregularly shaped particles can be calculated using Equation 1.

$$D_{ve} = \frac{D_m C_{ve}}{\chi C_m} \quad (1)$$

$D_{ve}$ is the volume equivalent diameter, $D_m$ is the mobility diameter, $\chi$ is the dynamic shape factor, and $C_{ve}$ and $C_m$ are the slip correction factors calculated for the volume equivalent diameter and mobility diameter, respectively. The volume equivalent diameter can then be used to calculate the particle's mass, as shown in Equation 2.

$$m_p = \rho_p \frac{4\pi}{3}\left(\frac{D_{ve}}{2}\right)^3 \quad (2)$$

$m_p$ is the mass of a particle and $\rho_p$ is the particle density. The aerodynamic diameter is defined as the diameter of a unit density sphere with the same settling velocity as the particle. The aerodynamic diameter describes a particle's aerodynamic behavior and is important in determining deposition in the respiratory tract. The aerodynamic diameter is calculated with Equation 3.

$$D_a = D_{ve}\left(\frac{\rho_p}{\rho_o \chi}\right)^{1/2} \quad (3)$$

$D_a$ is the aerodynamic diameter and $\rho_o$ is unit density. The dynamic shape factor used in these calculations was obtained from Slowik *et al*. (2004).

*Dose Calculation*

The dose deposited during the diesel exhaust ALI exposure is estimated from the deposition efficiency calculated from measurements with the test aerosol. The deposition efficiency (Equation 4) is calculated for each size bin ($i$) by comparing the total number deposited per cm$^2$ as measured with TEM with the total number of particles from the test aerosol that enter the cell enclosure during the exposure, as measured with the SMPS.

$$\text{Deposition efficiency}_i = \frac{\text{Number deposited}_i}{\text{Aerosol number concentration}_i \times \text{Flow rate} \times \text{Time}}\left[\frac{\%}{\text{cm}^2}\right] \quad (4)$$

The size distribution deposited in the ALI exposure is then calculated by multiplying the deposition efficiency for each bin with the total number of particles entering the cell enclosure during the diesel exhaust ALI exposure.

**TABLE 1**
**Particle Physical Characteristics**

| | Diesel | Flame |
|---|---|---|
| Fractal dimension | 1.61–1.87[a] | 1.65–1.82[b] |
| Primary particle diameter (nm) | 32[a] | 21 |
| Dynamic shape factor | 1.1–2.2[a] | 1.1–2.2[c] |
| Density (g/cm$^3$) | 1.3–1.78[a] | 1.8[c] |

[a]Data derived from Park *et al*. (2004).
[b]Data derived from Koylu *et al*. (1995).
[c]Data derived from Slowik *et al*. (2004).

## RESULTS

### *Deposition Measurement*

The size distribution of deposited test aerosol particles was determined by measuring the projected area diameter from the TEM images of 138 particles. Typical images of a test aerosol particle and a diesel exhaust particle from suspension are shown in Figure 2. The test aerosol particle displays the open fractal shape typical of combustion-generated particles, unlike the suspended diesel particle which has undergone aggregation due to the suspension process, resulting in a much larger diameter and more compact shape. Particles were binned by projected area diameter, corresponding to the SMPS mobility diameter bins. As shown in Figure 3, the flame generated test aerosol has a similar size distribution to that of the diesel exhaust from the ALI exposure. The number concentrations for the test aerosol and diesel exhaust size distributions are both approximately $1.0 \times 10^6$ cm$^{-3}$, but the diesel exhaust has somewhat larger particles with a mean diameter of 122 nm compared with 80 nm for the test aerosol. The deposited distribution is shifted to significantly larger diameters; the mean diameter of the deposited particles is 223 nm.

### *Diesel Particle Deposition*

The deposition efficiency and the diesel exhaust size distribution measured during the exposure at the ALI were used to calculate the dose that the cells received. The mass distribution is calculated for each size bin from their volume equivalent diameter (Equation 1) and a density of 1.8 g/cm$^3$

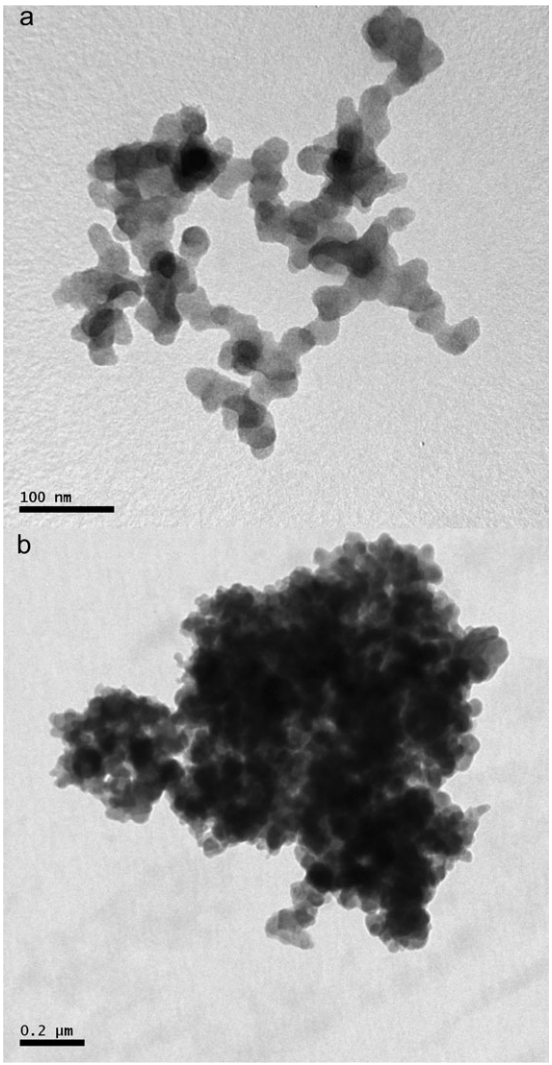


**FIG. 2.** TEM images of a typical (a) test aerosol particle, scale bar = 100 nm and (b) diesel exhaust particle, scale bar = 200 nm. The test aerosol particle was deposited on the TEM grid during the ALI deposition measurement. The diesel particle TEM sample was obtained by placing a drop of the diesel particle suspension on the TEM grid and wicking off the excess water with a filter paper.

(Slowik *et al.*, 2004). The total number and the total mass of particles deposited are $2.3 \times 10^7$ cm$^{-2}$ and $1.0 \times 10^{-4}$ μg/cm$^2$, respectively.

### *Comparison to Tracheal–Bronchial Deposition*

We compared the size distribution deposited during the ALI exposure with that in the tracheal–bronchial (TB) region of a human lung. The deposition in the human lung is calculated using an equation fit to the International Commission on Radiological Protection (ICRP) model of deposition in the TB region (Hinds, 1999). The ICRP model was developed from empirical and theoretical relationships predicting particle deposition in men and women at several levels of exercise. The ICRP deposition efficiency for the TB region and the same

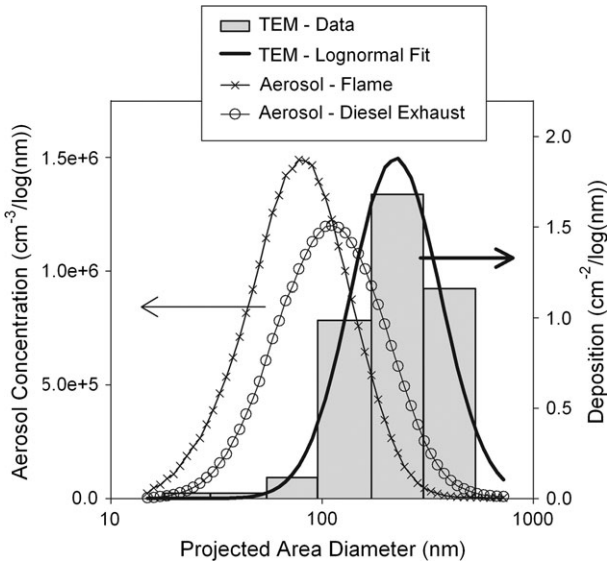


**FIG. 3.** Aerosol and deposited particle size distributions normalized by bin width. The flame and diesel particle size distributions are measured by the SMPS upstream of the cell enclosure and the deposited size distribution is measured from the TEM images of 138 particles in an area of 2420 μm$^2$ and fitted with a lognormal distribution.

diesel exhaust size distribution used to calculate the ALI dose are used to calculate the expected deposition in the lung. The predicted lung deposition is normalized by an estimated TB surface area of 2471 cm$^2$ (Mercer *et al.*, 1994). The estimated TB deposition is compared with the ALI deposition in Figure 4.

The deposition number in the TB region is $3.6 \times 10^7$ cm$^{-2}$, almost the same as the deposition at the ALI ($2.3 \times 10^7$ cm$^{-2}$). However, the distribution deposited in the TB region in the lung is skewed toward the smaller sizes, with a geometric mean diameter of 62 nm. This is much smaller than that observed at the ALI, which had a geometric mean diameter of 260 nm.

### *Cell Inflammatory Response*

Both the ALI and the particle suspension exposures decreased viability and increased IL-8 secretion. For the ALI exposure to whole or filtered exhaust, viability of both groups was reduced to approximately 75% of unexposed control cells. The IL-8 secretion of cells exposed to whole and filtered exhaust at the ALI were 311% and 230% of the unexposed cells, respectively. For the suspension exposure (Table 2), the viability was only appreciably decreased (94% of unexposed) for concentrations greater than 7.5 μg/ml. The IL-8 secretion was elevated for doses greater than 1.0 μg/ml and exhibited an increasing dose response.

To compare the ALI and the particle suspension exposure both the dose and the response were normalized by growth surface area. The cell viability and IL-8 secretion were normalized to baseline values to account for the growth area and exposure conditions. The baseline for the particle suspension exposure was the unexposed group. Because the ALI exposure

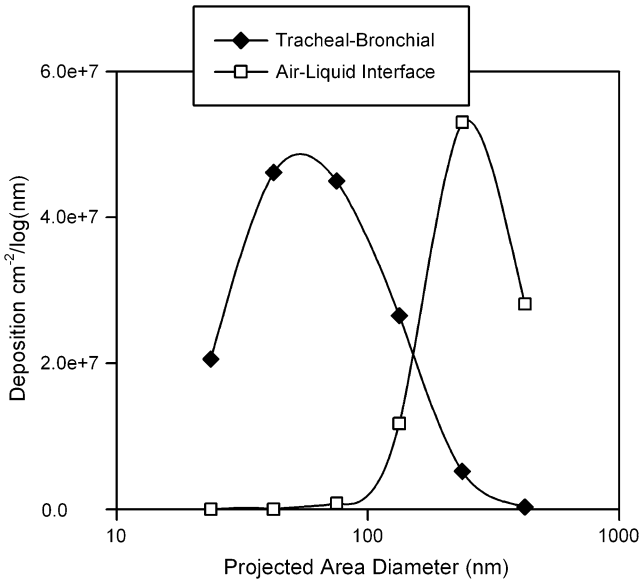


**FIG. 4.** Comparison of the estimated TB deposition with the estimated ALI deposition of the diesel exhaust particle size distribution (normalized by bin width). TB deposition is estimated using the ICRP model deposition fraction for the TB region with an average breathing rate of 0.5 $m^3$/h. ALI deposition is estimated using the size-dependent deposition fraction measured with TEM images of the flame test aerosol.

also included the effect of the exhaust gases, the baseline value was set as the filtered exposure. This baseline includes the exhaust gases, but not particles. In this way, only the effects of particles are compared between the two exposure methods, assuming that the effects of particles and gases are additive. The baseline normalized viability and IL-8 secretion are shown in Figure 5. The cellular response is similar, with viabilities of 96% and 94% and the IL-8 secretion of 136% and 148% of baseline for the ALI and 1.88 μg/$cm^2$ suspension dose, respectively. However, the doses producing these responses

**TABLE 2**
**Viability and IL-8 Release for *In Vitro* Exposure to Suspensions of Diesel Exhaust Particles**

| Dose, μg/ml (μg/$cm^2$) | Viability ± SEM (OD at 570 nm) | IL-8 ± SEM (ng/ml) |
|---|---|---|
| 0.0 (0.0) | 1.69 ± 0.03 | 1.66 ± 0.19 |
| 0.5 (0.13) | 1.65 ± 0.05 | 1.85 ± 0.09 |
| 1.0 (0.25) | 1.65 ± 0.04 | 1.81 ± 0.12 |
| 7.5 (1.88) | 1.59 ± 0.03 | 2.44 ± 0.13* |
| 10 (2.5) | 1.46 ± 0.03* | 2.93 ± 0.04* |
| 50 (12.5) | 1.45 ± 0.06* | 3.64 ± 0.11* |

*Note*. Cell viability was assessed using an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide based assay kit, and reported as the optical density (OD) of the solution at 570 nm. The concentration of IL-8 released was measured by commercially available ELISA and the measured values are corrected for viability. Values reported as mean and standard error of the mean, $n = 3$. *Statistically significant compared with control at $p < 0.05$.

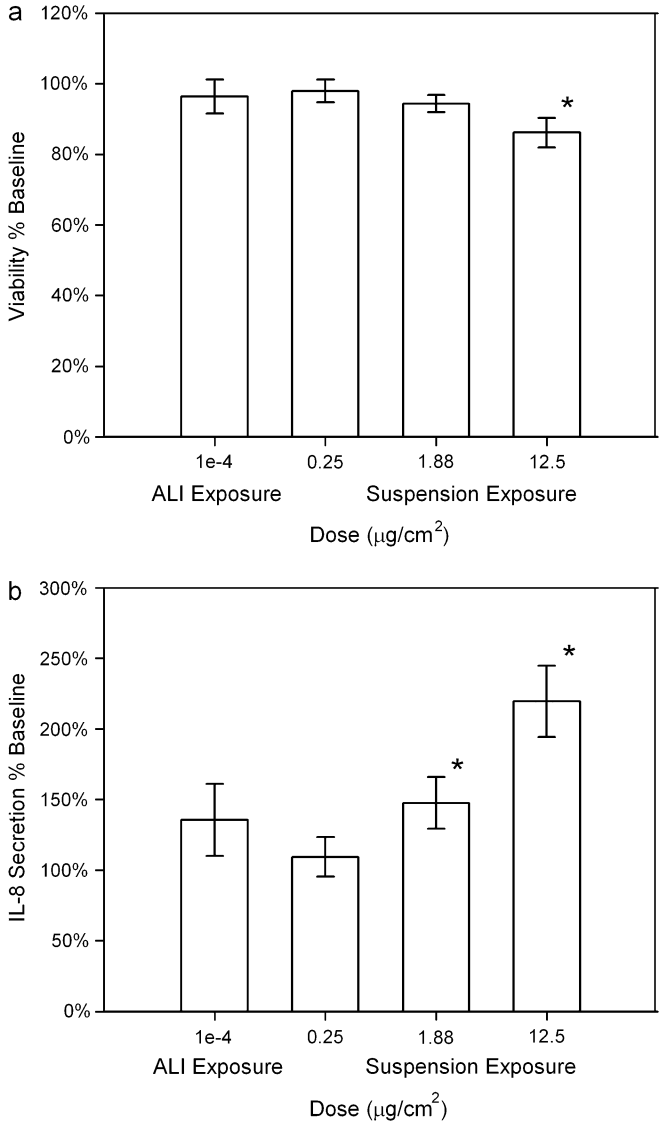


**FIG. 5.** Comparison of the (a) viability and (b) IL-8 release for ALI and suspension exposure to diesel exhaust particles. Viability and IL-8 values are reported as percent baseline, which for the ALI is the filtered group and for the suspension is control group. The concentration of IL-8 released was corrected for viability. The ALI baseline viability is 0.71 ± 0.04 and IL-8 secretion is 3.15 ± 0.31 ng/ml; the suspension baseline viability is 1.69 ± 0.06 and IL-8 secretion is 1.66 ± 0.32 ng/ml. Error bars represent the standard error of the mean, $n = 3$ for all groups except ALI viability which has $n = 4$. *Statistically significant compared with baseline at $p < 0.05$.

are very different—the suspension concentration dose is almost 20,000 times greater than the ALI dose of $1.0 \times 10^{-4}$ μg/$cm^2$.

## DISCUSSION

The ALI exposure was developed to provide an *in vitro* exposure to aerosol particles where the delivery to the cell is more like exposure of bronchial cells occurring during

inhalation than the conventional exposure of submerged cells to particle suspensions. Although the ALI exposure method has found increasing use, there is little information on how it compares to conventional *in vitro* exposures and lung deposition, which was the objective of our study.

One of the main advantages of the ALI exposure is that it can include the effects of gas phase species. This is useful for the assessment of combustion exhausts like diesel, which has many copollutants that also affect the cells. The effects of these gases are considerable, causing increased toxicity and IL-8 release in cells exposed only to exhaust gases (Holder *et al.*, 2007). In this study, the effects of the gases need to be separated from the particles to compare the ALI with the particle suspension exposure. These effects are removed by setting the ALI baseline as that of cells exposed to filtered diesel exhaust, assuming that the response to gas and particle components is additive. This is a cautious assumption, considering the evidence of synergistic interactions between particles and some exhaust gases (Lighty *et al.*, 2000).

The dose response of cells exposed to diesel exhaust at the ALI or to particles in suspension is dramatically different, with particles delivered at the ALI causing a similar response as particles in suspension at a dose several orders of magnitude greater. There are several possible sources for the different dose–response relationship observed between the suspension and ALI exposure, which are discussed below. With the suspension exposure, the particle's physical and chemical characteristics may be altered in the suspension process, semivolatile compounds may be lost during the sampling process, and the true dose to cells is not known. The ALI exposure-dose response may be affected by the culturing method and uncertainties in the dose measurement.

Surfactant coatings on particles suspended in medium, which help disperse hydrophobic particles, can dramatically change their toxicity. Coating with different chemicals has shown to be an important factor in quantum dot nanoparticle toxicity, usually decreasing *in vitro* toxicity (Hardman, 2006). The addition of DPPC reduced toxicity of suspensions of quartz dust to control level, but was less effective at reducing toxicity of asbestos (Schimmelpfeng *et al.*, 1992). However, coatings may have no effect or even increase toxicity, as diesel particles coated with DPPC exhibited similar genotoxicity as diesel particle extracts, though no direct comparison of coated and uncoated particles was made (Wallace *et al.*, 2007). Nickel ferrite nanoparticles coated with oleic acid were more toxic than uncoated particles (Yin *et al.*, 2005). Diesel particles also tend to aggregate in suspension, as demonstrated with the TEM images in Figure 2; this is enhanced by the high salt concentration of the cell growth medium. The aggregation can appreciably change the size distribution, shifting it to larger particle sizes. At high mass concentrations, aggregation can result in particles as large as the cells. Size-dependent effects may be another source of the different responses between the ALI and suspension exposures. These effects have been observed in particle phagocytosis. Depending on their size, particles were taken up by a different mechanism (Kreyling *et al.*, 2006).

It is difficult to establish a representative mass dose and size distribution in suspension exposures. Not all of the particles in the medium will settle onto the cell layer, resulting in a cell dose that is less than the medium concentration (Teeguarden *et al.*, 2007). The particles rapidly aggregate in the growth medium, and the larger particles settle to the cell surface, but smaller particles may remain suspended in the medium. These small particles do not contact the cell and thus may not contribute to the true cellular dose. However, as can be seen in Figure 2, diesel exhaust particles in suspension aggregate into large particles that contain the majority of particle mass, which will settle onto the cell layer. Therefore, the medium concentration is a reasonable estimate of the true cell mass dose. There are also some uncertainties in the dose deposited at the ALI due to differences of the test aerosol from the diesel particles (Table 1). The diesel particles have a lower density that further decreases with decreasing particle size (Park *et al.*, 2004). However, the density difference is small for the large particles most affected by gravitational settling, and is expected to cause a minimal overestimation of deposition (about 5%). The smaller diesel particles which have much lower densities than flame generated particles will have similar deposition efficiencies because they are deposited primarily by diffusion, which is independent of density.

Combustion particles have many semivolatile organic compounds adsorbed onto the surface that can be lost during collection and processing. In measurements of ambient particles, it was found that approximately 70% of the organic carbon on the particle can be lost during sampling (Eatough *et al.*, 1989). These semivolatile compounds are made up of a variety of hydrocarbon species, such as lighter weight PAHs, which can cause a proinflammatory response (Kawasaki *et al.*, 2001). Using an oxidation catalyst on diesel exhaust that removed 60% of PAHs, reduced cytokine release from exposed cells by 90% (Boland *et al.*, 1999). We previously investigated the effects of the gas phase and some of the semivolatile hydrocarbon compounds by removing them with an activated charcoal denuder. Removing these species from the exhaust of an unloaded engine decreased the IL-8 secretion almost to baseline levels, showing the importance of these compounds in the inflammatory response observed *in vitro* (Holder *et al.*, 2007). Because a portion of these compounds has been lost during collection and processing, the suspended particles will have decreased potency compared with the particles in the ALI exposure.

The culture method may also play a role in the different responses between the ALI and conventional suspension exposure. Changing from a submerged to an ALI culture has been shown to increase oxidative stress (Kameyama *et al.*, 2003). Although the effects of being grown in the ALI configuration are accounted for by using an appropriate

control, the already stressed anti-oxidant defenses may make the cells more susceptible to the effects of the particle exposure.

Because the delivery to the ALI is passive, particles are deposited on the cell layer by diffusional and gravitational forces. Diffusion is only important for small particles, less than 40 nm, which are present in the aerosol at minimal concentrations. Gravitational settling increases with particle diameter, therefore the larger particles settle at greater rates than smaller particles. These deposition mechanisms result in a distribution skewed to the larger sizes, as displayed in Figure 3.

Although ALI exposures are skewed toward larger particles compared with the TB deposition (Fig. 4), this is a considerable improvement over the conventional particle suspension exposures where particles rapidly aggregate into large particles. For example, in the suspension exposure at the highest dose, some particles were the same size as the cells (about 10 μm). The ALI and TB deposition number concentrations are approximately the same. Impaction type ALI exposures have modeled depositions one to several orders of magnitude greater than our passive exposure method, depending on the particle size (Desantes *et al*., 2006; Tippe *et al*., 2002). Also, impaction exposure has high deposition efficiencies for large and small particles, but minimal efficiencies for medium sized particles (50–500 nm); and particles tend to localize in an area in the center of the impaction plane (Desantes *et al*., 2006). The horizontal ALI exposure like that used by Lestari *et al*. (2006), where the flow is parallel to the cell surface may have enhanced deposition of small particles, but the deposition has yet to be fully characterized. It is possible that particles following the streamline will be close to the cell layer for a longer time allowing greater diffusion to the surface and higher deposition efficiencies for the smaller particles. This may also be more representative of the conditions in the TB region of the lung, where the inhaled air flows over the epithelium at Reynolds numbers ranging from 4800 down to about 2 (Hinds, 1999).

Ultimately, the goal of the ALI is to achieve an exposure that is similar to that of a human breathing under relevant exposure conditions. In our system, particles in the ultrafine size range (less than 100 nm) are deposited less efficiently than larger particles. Gravitational settling decreases with decreasing particle size and although diffusional deposition increases for smaller particles, the deposition efficiencies for ultrafine particles are still lower compared with that of particles larger than 100 nm. It is important to capture this size range because of the great interest in the toxicological effects of nanoparticles, with their unique physical properties and interactions with biological systems (Oberdorster *et al*., 2005). Furthermore, the ultrafine particles dominate the ambient number concentrations, and have a high deposition in the human respiratory tract (Oberdorster *et al*., 2005). The composition of urban ambient particles is also size dependent, with the ultrafine particles being derived primarily from combustion sources (Hinds, 1999). Although not definitive, there is some evidence of an association between morbidity and ambient ultrafine number concentration (Penttinen *et al*., 2001). Optimization of the ALI exposure to better capture this size range will improve its potential to be an effective rapid assay that can be used to assess toxicity and predict associated human health effects.

## CONCLUSION

The ALI exposure method has proven to be a more sensitive *in vitro* exposure; it exhibits a similar response to the conventional particle suspension exposure at a dose four orders of magnitude lower. Detailed information on the dose size distribution can be obtained with ALI exposure, unlike particle suspensions where the dose and size distribution that cells are exposed to are different from the medium concentration. Furthermore, with ALI, cells are exposed to aerosolized particles avoiding artifacts introduced by the collection and suspension processes. The number of particles depositing at the ALI is similar to that in the TB region of the lung, but composed of larger particles. Further optimization of the ALI exposure method is needed to achieve a size distribution that is more representative of that deposited in a human lung.

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