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Toxicity Assessment of Air-delivered Particle-bound Polybrominated Diphenyl Ethers

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Abstract

Human exposure to polybrominated diphenyl ether (PBDE) can occur *via* ingestion of indoor dust, inhalation of PBDE-contaminated air and dust-bound PBDEs. However, few studies have examined the pulmonary toxicity of particle-bound PBDEs, mainly due to the lack of an appropriate particle-cell exposure system. In this study we developed an *in vitro* exposure system capable of generating particle-bound PBDEs mimicking dusts containing PBDE congeners (BDEs 35, 47, 99) and delivering them directly onto lung cells grown at an air-liquid interface (ALI). The silica particles and particle-coated with PBDEs ranged in diameter from 4.3 to 4.5 μm and were delivered to cells with no apparent aggregation. This experimental set up demonstrated high reproducibility and sensitivity for dosing control and distribution of particles. ALI exposure of cells to PBDE-bound particles significantly decreased cell viability and induced reactive oxygen species generation in A549 and NCI-H358 cells. In male Sprague-Dawley rats exposed *via* intratracheal insufflation (0.6 mg/rat), particle-bound PBDE exposures induced inflammatory responses with increased recruitment of neutrophils to the lungs compared to sham-exposed rats. The present study clearly indicates the potential of our exposure system for studying the toxicity of particle-bound compounds.

Keywords

Particle-bound PBDEs; Lung cells; Air-liquid interface; *In vitro* toxicity testing; Cytotoxicity

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Conflict of Interest
The authors declare no conflict of interest.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are commonly used as flame retardants that are added to a wide variety of consumer products, such as upholstered furniture, carpet, building materials, toys and electronic goods (Allen et al. 2008; Birnbaum and Staskal 2004; Harrad et al. 2006). Since 2004, the penta- and octa-brominated diphenyl ether mixtures, two of the three main commercial formulations, have been banned in the European Union (EU) and voluntarily phased out of the USA market because of concerns over the persistence and toxicity of PBDEs. Deca-BDE, the third main technical formulation, is currently being phased out in the EU and its production, importation and use in the USA will cease by the end of 2013 (EPA 2010). Despite efforts to ban commercial PBDE mixtures, PBDEs will remain in the environment and in biological matrices because of their persistence and ability to bioaccumulate. Thus, human exposure to PBDEs will likely continue for decades similar to polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) even if their production and use are discontinued (Watkins et al. 2011).

PBDEs are persistent, bio-accumulative and have some structural similarities to PCBs and PBBs that can disrupt the immune, reproductive, nervous and endocrine systems in animals (EPA 2010; Gao et al. 2009; He et al. 2009). PBDEs interfere with the endocrine system (thyroid hormone), (Ren et al. 2013) impair neurobehavioral development (Dingemans et al. 2011; He et al. 2009) and induce DNA damage (Gao et al. 2009; He et al. 2008; Lai et al. 2011) in animals and human cells *in vitro*. Data show that BDE47 and BDE99 disturb the development of primary fetal human neural progenitor cells *in vitro* via disruption of cellular thyroid hormone signaling (Timm Schreiber 2010). Co-exposure to BDE47 (1-2.5 μM) and BDE99 (5-30 μM), in particular at low doses, induced synergistic oxidative stress-mediated neurotoxicity in human neuroblastoma cells (SK-N-MC cell lines) (Tagliaferri et al. 2010). An *in vitro* study showed that BDE47 (4 μg/mL) inhibited cell viability, increased lactate dehydrogenase (LDH) leakage, induced reactive oxygen species (ROS), DNA damage and cell apoptosis in human neuroblastoma (SH-SY5Y) cells (He et al. 2008).

PBDEs are not permanently bound to the products and can be released from the products into the environment as dust (particle-bound) or as vapor (de Wit 2002). Therefore, PBDEs have been commonly detected in indoor air, house dust and human tissues such as serum and breast milk (Allen et al. 2006; Batterman et al. 2009; Schecter et al. 2003; Vorkamp et al. 2011). Human exposure pathways to PBDEs remain unclear, even though the indoor environment is an important source of exposure to PBDEs used in household products (Allen et al. 2008; Harrad et al. 2006; Vorkamp et al. 2011). The main routes of human exposure to PBDEs appear to occur *via* food consumption, ingestion of dust and inhalation of PBDE-contaminated air and particle-bound PBDEs, principally in indoor exposure scenarios (Harrad et al. 2006; Huwe et al. 2008; Vorkamp et al. 2011; Wilford et al. 2008). PBDEs were found at high concentrations in house dust (BDE47 and BDE99 were 16.9 and 13.6 ng/g, respectively) and residential indoor air (BDE47 and BDE99 were 134 and 63.7 pg/m^3, respectively) (Vorkamp et al. 2011). It has been widely accepted that indoor air and dust concentrations were higher in North America than in continental Europe (Frederiksen et al. 2009). BDE47 and BDE99 were the dominant congeners in indoor air and dust collected from USA urban residences as well as in human tissues (Allen et al. 2006; Batterman et al. 2009; EPA 2010). Interestingly, strongly elevated blood levels of PDBE among aircraft crew and passengers were associated with inhalation exposures (Christiansson et al. 2008). Inhaled PBDEs in dust and corn oil were readily bioavailable and are biologically active in male rats, as indicated by increased transcription of hepatic enzymes. PBDEs and structurally similar semi volatile organic contaminants, such as PCBs and PAHs, are more enriched in the fine indoor particles than coarse particles. Chemicals bound to smaller

particles are more bioavailable and have a longer pulmonary residence time (Hwang et al. 2008; Meeker et al. 2009; Paustenbach et al. 1997; Shoeib et al. 2012). These observations support the significance of dust in exposure to particle-bound contaminants.

Few studies have examined pulmonary toxicity of particle-bound PBDEs using *in vitro* models mainly due to the lack of an appropriate particle-cell exposure system. In some experimental designs, particles are directly added to the cell culture medium for the assessment of particle toxicity. However, these approaches have limitations, including poor reproducibility, changes of particle size due to the aggregation, interactions of particles with components of the medium (e.g., albumin), and dissolution of particles by the medium (Fatisson et al. 2012; Savi et al. 2008). These limitations may account for poor correlation between toxicity of particle-types tested by *in vivo* insufflation versus *in vitro* cell culture exposures (Sayes et al. 2007). Differences in cell types, media compositions, exposure concentrations, and particle delivery techniques make comparisons between *in vitro* toxicity studies difficult.

Inhaled particles first interact with pulmonary surfactants, which are produced by epithelial type II cells and cover the alveolar region to prevent alveolar collapse among other functions. Coating with surfactant may alter surface characteristics and subsequent toxicity of the inhaled particle. The most commonly used *in vitro* model for assessing pulmonary toxicity of inhaled particles, the liquid cell culture method does not replicate the *in vivo* conditions of lung cells. In contrast, air-liquid interface (ALI) models more closely reflect *in vivo* conditions by providing an air-facing surface of epithelial cells with a thin layer of airway surface liquid at the air interface (Jayaraman et al. 2001). Moreover, epithelial cells grown at the ALI have well-differentiated structures and functions compared to cells grown immersed in the medium (Kameyama et al. 2003). These features account for the increase in the use of ALI models for the *in vitro* toxicity study of particles (Bitterle et al. 2006; Kim et al. 2013; Lenz et al. 2009; Savi et al. 2008; Stringer et al. 1996; Tippe et al. 2002).

The goal of this research was to develop a realistic inhalation exposure model for airborne particle-bound PBDEs. We first coupled an ALI cell exposure system with a particle aerosolizer and exposure chambers allowing the airborne particles to directly interact with the lung cells cultured on commercially available semipermeable membranes. A preliminary study was carried out to validate the reproducibility of the distribution and dose of particles and the impact on biological endpoints, cytotoxicity and ROS using two types of human lung cell lines (A549 and NCI-H358) as a precursor to studies of particle-bound PBDE toxicity. We also evaluated pulmonary responses of the same PBDE-bound particles delivered to rats *via* intratracheal insufflation to compare the results from *in vitro* studies to *in vivo* toxicity.

2. Material and methods

2.1. Materials

Three PBDE congeners—3,3',4-tribromodiphenyl ether (BDE35), 2,2',4,4'-tetrabromodiphenyl ether (BDE47), and 2,2',4,4',5-pentabromodiphenyl ether (BDE99)—were chosen as study compounds on the basis of their prevalence, origin and toxicity (Klo□sener et al. 2008). BDEs 35, 47 and 99 were synthesized by nucleophilic aromatic substitution of the diphenyliodonium salts with bromophenols as described previously (Klo□sener et al. 2008). A spherical silica gel particle (Nucleosil, pore size 100 Å, Macherey-Nagel, Inc., Bethlehem, PA USA) was used as the carrier material.

2.2. Cell culture

Two types of human lung cells (A549 and NCI-H358, American Type Culture Collection, Manassas, VA, USA) were used to evaluate cellular responses of particle-bound PBDEs. A549 cells are transformed human type II alveolar epithelial cells with endocytic properties. Type II cells continuously regenerate the airways epithelium (Stringer et al. 1996). NCI-H358 cells are Clara (club)-type cells that detoxify harmful substances inhaled into the lungs, being one of the few lung cell types in which cytochrome P-450 enzymes are expressed. Cells were cultivated in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and 100 mg/mL penicillin and streptomycin (Invitrogen). The cells were grown in T-cell culture flasks (75 cm^2 of growth area) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed 24 h after subculturing and every 2 days thereafter (up to passage 30). The cells were split 1:10 during each passage.

2.3. Preparation of particle-bound PBDEs

PBDEs are often applied as flame-retarding coatings to a parent backbone material that may range from an inorganic material to an organic polymer (de Wit 2002). PBDEs from these products are then released into the air by volatilization. We therefore generated and characterized coatings of our PBDE congeners on silica particles (Nucleosil) as a model of airborne particle-bound PBDEs to mimic inhalation exposure of mixtures of dust and PBDEs. Spherical silica particles coated with PBDEs were generated as described previously (Klo□sener et al. 2008). Briefly, for the *in vitro* study, a stock solution of individual PBDE congeners (BDEs 35, 47 or 99) was prepared for each PBDE congener to a concentration of 2 mg/mL diethyl ether. Spherical silica particles (as support materials, 40 mg), stock solution (5 mL) and diethyl ether (5 mL) as a solvent were mixed in a round bottom flask (each PBDE congener represented 20% of the weight of the silica particles). The solvent evaporated under reduced pressure in a rotary evaporator with tumbling at 30 rpm and outside warming to 36°C.

The *in vivo* study a stock solution was prepared with a total PBDE concentration of 2 mg/mL diethyl ether, containing equal molar amounts of each congener, BDEs 35, 47 and 99. The PBDEs represented 20% of the weight of the silica particles. The silica particles (40 mg), stock solution of PBDE mixture (5 mL) and diethyl ether (5 mL) were mixed together as described above.

2.4. In vitro exposure system for air-delivery of particle-bound PBDEs

An *in vitro* exposure system was designed for assessing the toxicity of air-delivered particle-bound PBDEs to human lung cells. Figure 1 shows a cross-section representation of our *in vitro* exposure system. The exposure model consists of two major chambers: (1) a delay chamber to mix particles with air, reduce particle interactions with the chamber (e.g., electrostatics) and thus increase uniform distribution of particles; (2) an exposure chamber holding a semi-permeable cell culture membrane (12 mm of diameter, 1.1 cm^2 of growth area, Corning, Inc., NY, USA) to deposit particles to lung cells at an ALI. A dry powder insufflator (model DP-4, Penn-Century, Inc., PA, USA) with an air pump (AP-1, Penn-Century, Inc.) was used to aerosolize particle-bound PBDEs into the delay chamber. Clean air from a laminar flow hood was drawn by an external air pump and passed through the delay chamber where it was combined with the particle aerosol stream and fully mixed, and further into the exposure chamber at 10 mL/min. Cells grown on the transwell membrane were exposed to particle-bound PBDEs *via* the air at the apical surface in the exposure chamber.

2.5. Characterization of the in vitro exposure system

To validate and characterize the exposure system, the size, morphology and spatial variability of particles (particle distribution) were determined by scanning electron microscope (SEM, Hitachi S-4800 FE, Tokyo, Japan) and image analysis (ImageJ, NIH, USA). To evaluate the size and morphology of air-delivered particles in the exposure system, silica particles and PBDE-coated silica particles (0.45 ~ 0.65 mg) were aerosolized and deposited onto cell-free transwell membranes treated with culture medium (100 μL) at a flow rate of 10 mL/min for one minute after spraying of the particles into the delay chamber with the dry powder insufflator with air pump. The membranes were then removed from the transwells with a clean scalpel blade. Samples were allowed to air dry, and the membranes of the transwells were mounted on an aluminum SEM stub with double-sided, graphite tape and the edge of the filter was painted with colloidal silver liquid to promote conductivity. The surface of the sample was sputter-coated with gold (K550, Emitech, Ashford, Kent, England) and examined under SEM by observing size and morphology of particles.

To determine the distribution of particles, the membranes were attached to a gridded microscope slide with double-sided tape and photographed at different distances from center of the transwell membrane using a light microscope. The images of particles deposited on the membranes were characterized by ImageJ software (number of particles/2.5 × 10^{-4} cm^2 of image area) to estimate the total number of particles on the transwell (cell growth area, 1.1 cm^2). Poisson distribution was used to compute the number of particles deposited within the cell growth area. Particle dosing efficiency was determined by correlation between particle loading mass (0.45 ~ 0.65 mg) in particle aerosolizer and surface cover ratio of particle area versus cell growth area.

2.6. Air-delivery of particle-bound PBDEs to lung cells

For ALI exposure experiments, cells were harvested with 0.25% trypsin-ethylenediamine tetraacetic acid (Trypsin-EDTA, Invitrogen), counted and seeded at a density of 1.5×10^4 cells/cm^2 onto 1.1 cm^2 commercially available transwell membranes (0.4 μm, Transwell, Corning). Cells were allowed to attach to the transwell for 12 h. When cells had grown to confluence (> 85% surface covered), they were cultured at the ALI for 12 h prior to exposure to particles. Cells were then washed twice with phosphate buffered saline (PBS) and transferred to the exposure chamber where they were exposed to particle-bound PBDEs at the ALI. Two different mass concentrations of particle-bound PBDEs (28 and 45 μg/cm^2) were directly delivered to both A549 and NCI-H358 cells at a flow rate of 10 mL/min for one minute. Airborne exposure of lung cells grown at the ALI was employed to mimic the exposure conditions of epithelial lung cells *in vivo*. Intracellular ROS and cytotoxicity were determined 4 and 24 h after exposure. The exposure time points for ROS (4 h) and cell viability (24 h) assays were based on previous studies (He et al. 2008; Jin et al. 2010; Tagliaferri et al. 2010) with the assumption that increased oxidative stress may be an early event and cytotoxicity a later event.

2.7. Submerged cell exposure to PBDEs

To facilitate comparison of cellular responses to air-delivered particle-bound PBDEs at the ALI with responses in submerged cultures, PBDE exposures were also performed using a conventional cell culture/PBDE in solution method. For submerged cell exposures, cells (1×10^5 cells/cm^2) were seeded in complete medium into flat-bottom polystyrene 48-well plates (BD Falcon, Franklin Lakes, NJ, USA) and grown to approximately 85-90% of confluency. Cells were allowed to adhere to cell culture plates for 24 h prior to exposure. PBDEs were dissolved in dimethyl sulfoxide (DMSO) and then diluted with PBS with Ca^{2+} and Mg^{2+}. Before treatment with the compounds, cells were washed with PBS and then exposed to the compounds (5 μM, 10 μM, 20 μM, 40 μM and 80 μM, final

concentration) in complete cell culture medium for 24 h at 37 °C in humidified air with 5 % CO_2. After exposure, cytotoxicity and intracellular ROS were determined immediately. Control cultures received solvent (DMSO, 0.1% final concentration) alone.

2.8. Cytotoxicity and intracellular ROS measurement

Cell viability, as an indicator of cytotoxicity, was determined by Alamar Blue assay (Serotec, Oxford, UK), a method that is commonly used to assess mitochondrial function. Five mM A amar B ue stock in PBS was di uted to 50 μM with medium. After the incubation period (24 h), cells were washed twice with PBS, fresh cell culture media containing 50 μM A amar Blue was added and cells were incubated for 1 h at 37°C in humidified air with 5% CO_2. The fluorescence was monitored at 535 nm excitation and 595 nm emission wavelengths using a TECAN microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

A fluorescent probe, DCFH-DA (2',7'-dichloro-difluorescin diacetate, Molecular Probes, OR, USA) was used to measure intracellular ROS. DCFH-DA is permeable across cell membranes and is hydro yzed in ce s to 2',7'-dichlorofluorescin (DCFH). DCFH is then oxidized to 2',7'-dichloro-fluorescein (DCF) by ROS (Gomes et al. 2005). The fluorescence dye was dissolved in dimethylformamide (DMF) to make a 5 mM stock solution and diluted with Hanks Balanced Salt Solution (HBSS, Invitrogen, Grand Island, NY, USA) to make a 5 μM working solution. Four hours after particle-bound PBDE exposure or exposure to PBDE in solution, cells were washed with HBSS and incubated in HBSS containing 5 μM DCFH-DA for 1 h in the dark at 37° C. DCF fluorescence was measured at 485 nm excitation and 535 nm emission wavelengths using a TECAN microplate reader (Tecan Group Ltd.).

2.9. Animals and intratracheal insufflation

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were exposed to silica (n=2/group) or PBDE-coated silica particles (n=2/group) acutely *via* intratracheal insufflation at a dose of 0.6 mg/rat. Protocols were approved by the Institutional Animal Care and Use Committee and complied with the NIH Guide for the Care and Use of Laboratory Animals. Animals were acclimatized after arrival for 12 days prior to use. They were housed in an AAALAC-accredited vivarium in polypropylene cages and supplied with feed, a standard rodent chow, water *ad libitum*, and maintained on a 12-hr light-dark cycle.

Animals were anesthetized by 5% isoflurane inhalation using a precision Fortec vaporizer (Cyprane, Keighley, UK) and prior to intratracheal insufflation with a dry powder insufflator (model DP-4, Penn-Century, Inc.). After exposure, the animals were euthanized by isoflurane inhalation at 2 h, 4 h, 8 h, 12 h, 16 h and 24 h post exposure (silica-exposed animals, shams were euthanized 8 h after exposure) and each lung was lavaged 3 times (total volume, 15 mL) with 0.9% sterile sodium chloride solution (Baxter, Deerfield, IL). Bronchoalveolar lavage (BAL) fluid was collected, processed and the cell pellet was used for enumeration of total and differential cells. Lungs were fixed in 10% formaldehyde-phosphate-buffered saline solution *via* the canulated trachea. The tissues were subsequently paraffin-embedded, sectioned at 5 μm, and stained with hematoxylin and eosin (H & E) for histopathology evaluation.

2.10. Statistical analysis

All *in vitro* experiments were conducted in triplicate. Cells exposed to PBDE-free silica particles in ALI exposure conditions or cells exposed to DMSO alone in submerged cell culture conditions were used as controls. The values of cells exposed to particle-bound PBDEs at the ALI or PBDEs in solutions were normalized to control. Statistical analyses

were performed using R software (R Development Core Team 2008). Poisson distribution was used to compute the number of particles depositing on transwell membrane, (#/cm^2) at 95% confidence intervals using the *in vitro* exposure system. A *p*-value less than 0.05 was considered statistically significant. All data are expressed as means ± standard error (SE) unless otherwise noted.

3. Results

3.1. Characterization of the in vitro exposure system

The size silica particles used as support materials and particles coated with PBDEs ranged in diameter from 4.4 to 4.6 μm (Figure 2A). Particles presented as discrete spheres with no apparent particle aggregation after deposition on the transwell membrane in the exposure system (Figures 2B and C). As shown in Figure 3, particle deposition was highest at a distance 2 mm from center and decreased along one side of the membranes. The SEM images also show that the particles were quite evenly deposited over the membrane at 2 mm (Figure 2B) and 2.8 mm (Figure 2C) from center.

The Poisson distribution was used to compute the total number of particles deposited on the transwell membranes to evaluate the particle dosing efficiency in our exposure chamber. As shown in Table 1 and Figure 4, particle number deposited strongly correlated with particle mass loading (R^2=0.97). The surface covered by particles ranged from 27 to 42% of the growth area, depending on the dose of particles (Figure 4).

3.2. Cellular responses to air-delivery of particle-bound PBDEs

As shown in Figure 5A, 24 h after ALI exposure to PBDE-coated particles, statistically significant decreases in the viability of A549 cells were observed in response to doses of 28 and 45 μg BDE35-coated particles (p<0.001 and p<0.05, respectively) compared to control cells that were exposed to silica particles alone. A significantly decreased viability of A549 cells at a dose of 28 μg BDE47-coated particles (p<0.01) was found, whereas BDE99-bound particles did not significantly decrease cell viability. No significant reduction in the viability of NCI-H358 cells in all treatment groups.

As shown in Figure 5C and D, 4 h after ALI exposure, a significant increase in ROS in both cell lines was detected in response to the doses of 28 and 45 μg BDE35-coated particles (p<0.05) compared to control (silica particles alone). BDE47-bound particles significantly increased the intracellular ROS in A549 (at 45 μg, p<0.001) and NCI-H358 cells (at 28 μg, p<0.05). ALI exposure of cells to BDE99-bound particles significantly increased ROS in A549 (at 45 μg, p<0.001) and NCI-H358 cells (at 28 μg, p<0.05).

3.3. Cellular responses to PBDEs in solution exposure system

As shown in Figure 6, the viability of A549 cells treated with BDE47 was significantly lower only at a concentration of 40 μM (p<0.01) and cell viability following exposure to BDE99 decreased at concentrations of 20 (p<0.01), 40 (p<0.05) and 80 μM (p<0.05). The levels of intracellular ROS in A549 cells (Figure 6 C) were examined at 4 h after PBDE treatment in solution. A significant increase in ROS was detected in response to BDE35 at 5 (1.3-fold), 10 (1.3-fold) and 20 μM (1.2-fold) compared to control (DMSO alone). Exposure of cells to BDE47 resulted in significant increases in ROS at 5 (1.4-fold) and 10 μM (1.5-fold) compared to control. BDE99 produced a significant increase in ROS from 1.4-, 1.5- and 1.5-fold at concentrations of 5, 10 and 20 μM, respectively. In NCI-H358 cells exposed to 5 μM BDE47 and all concentrations of BDE99, the intercellular ROS level was significantly increased compared to the control (p<0.05, Figure 6D).

3.4. Intratracheal insufflation

To evaluate pulmonary responses to PBDE-coated particles, we examined BAL fluids and lung histopathology in rats exposed acutely to silica particles or PBDE-coated particles at different post-exposure time (Figure 7). The number of macrophages in BAL fluids from animals exposed to PBDE-coated particles significantly increased over time, reaching a peak at 8 h post-exposure, $p < 0.05$, and then slowly decreasing thereafter (Figure 7A). A significant increase in the number of neutrophils over time was observed also reaching a maximum at 8 h post exposure, $p < 0.05$, and then slowly declining thereafter. PBDE-coated particles caused a significant inflammation in the lungs represented by a high recruitment of neutrophils (70% + 4), that were slowly decreasing to 41% (+ 12) at 24 h after exposure (Figure 7A). The total number of cells in the BAL fluid at the 24 h time point was approaching the number of cells in shams at the 8 h time point.

Histopathologic evaluations of lung tissues showed neutrophilic peribronchiolar infiltration (at 8 to 16 h post-exposure) that became less severe with a mixed inflammatory cell population by 24 h post-exposure (Figure 7B and C). Within the airways and the terminal bronchiolar-alveolar duct junctions, there were focal accumulations of macrophages, often containing phagocytosed particles, and small number of neutrophils was most prominent at 8 to 16 h post-exposure to PBDE-coated particles (data not shown). Phagocytosed particles in PDBE-exposed rats appeared as round, pinkish, slightly refractile spheroids within macrophages or neutrophils which had severely peripheralized nuclei, but particles were not observed in the cells of silica only treated rats.

4. Discussion

We generated and characterized coatings of PBDE congeners on silica particles as a model of airborne particle-bound PBDEs to mimic inhalation exposure of mixtures of dust and PBDEs. The *in vitro* exposure model employing an ALI was built to assess potential pulmonary toxicity of these particle-bound PBDEs. Our results demonstrate that the exposure system fulfills several important requirements. First, the system is able to be used to reproducibly generate and deliver particles with no apparent aggregation and cell damage by airflow. The size of the spherical silica and PBDE coated particles is in the range of respirable particles (smaller than 3 μm in adults and 5 μm in children) (Brown et al. 2013), including dusts that contain PBDEs. Second, particles were evenly deposited over the membrane except that relatively fewer particles were deposited at the outer edge of the membrane (at 4.5 mm from the center). This decrease was likely due to the outer diameter (4 mm) of the aerosol delivery tube being smaller than that of the transwell membrane and the higher velocity of the outer particles (Desantes et al. 2006; Tippe et al. 2002). An uneven distribution of particles could cause artifactual, localized, cellular responses in overexposed areas on the transwell due to excessive localized particle loading. Our results show that the risk of such uneven responses was relatively small in our exposure set-up. Third, the exposure system is able to be used to provide an ALI for lung cells to mimic airborne exposure and avoid interactions between particles and culture medium. Our results demonstrate that the new *in vitro* exposure system allows for dosing and surface cover ratio control of particles.

We recognize that choosing the appropriate endpoints and cell types based on the biological effects of a test substance and biology of the target organ is a key to the establishment of *in vitro* toxicity systems. Non-small lung carcinoma cell lines (A549 and NCI-H358) are widely used to study the toxicity of airborne particles in lung. While primary human lung epithelial cells may be a better choice for the toxicity assessment of inhalable particles, primary cells are more difficult to obtain and have limited growth potential. A main goal of this work was not to elicit mechanisms in responses to particle-bound PBDEs, but focus on

characterization and validation of the system. To validate biological aspects of the exposure system, we chose the viability and intracellular ROS generation of lung cells in responses to air delivered particle-bound PBDEs as initial endpoints. Selecting endpoints related to the changes of signaling pathways is, therefore, needed for future work since PBDEs exert their toxicity through interfering with intracellular signaling pathways. Statistically significant decreases in cell viability in response to PBDEs under solution cell culture conditions were of limited biological importance since reduction ranged only from 6 to 9%. The pattern of cytotoxic effects caused by PBDEs in NCI-H358 cells was similar under both exposure conditions, indicating a lower sensitivity of this cell line to these compounds. Exposure of lung cells from each cell line to 5 μM to 80 μM PBDE (congeners 35, 47 or 99) in the solution exposure method was not associated with a dose-dependent effect on cytotoxicity or intracellular ROS. The main reason for this observation is likely the partitioning of PBDEs into the medium under solution culture conditions. A recent investigation (Barber et al. 2006) of the partitioning of PBDEs from media into MCF-7 cells under solution cell culture conditions reported that only 5.5 and 1.9 % of BDE47 and 99, respectively, partitioned into the cells. They suggested that large losses occur during incubation with PBDEs, apparently adsorbed onto the plastic, resulting in the cells being exposed to lower doses than intended. Given these issues with the solution culture method, the biological effects of particle-bound PBDEs would not be accurately evaluated using this method. The ALI delivery method developed in this work may have the additional advantage of circumventing this problem.

Overall ROS was a more sensitive endpoint than cytotoxicity and A549 cells (epithelial cells) seemed to be more sensitive than NCI-H358 cells (Clara cells) to PBDEs in this study. BDE35 induced toxicity in A549 and ROS in NCI-H358 cells only if delivered as particles, while BDE99 induced cytotoxicity in both cell types only if applied in solution. These results indicate that there are cell types, test compounds and delivery specific effects, which can be further employed to obtain insights into physicochemical and biological determinants of *in vitro* toxicity. Disparities in cellular responses between lung cell lines and primary lung cells are common (Courcot et al. 2012; Melis et al. 1996; Nirmalanandhan et al. 2010). Lung surfactants and metabolic capacities of lung epithelial cells may alter toxicity of the inhaled particles and cell lines used in this study have specific cellular features such as high levels of glutathione and gene expression of heme oxygenase-1 (A549) (Carmichael et al. 1988; Speit and Bonzheim 2003) and expression of lung surfactant associated proteins and cytochrome P-450 enzymes (NCI-H358) (Gazdar et al. 1990; Hasenpusch et al. 2011). These differences may represent a possible explanation as to why A549 cells were more vulnerable to toxicity of PBDE-bound particles compared to NCI-H358 cells, but this warrants further investigation. Indeed, a recent study (Courcot et al. 2012) reported that A549 and NCI-H358 cell lines exhibited distinct differences in gene expression patterns (Pearson's correlation coefficient, r=0.75; the r value represents the strengths of the linear relationship between two sets of comparative components, a greater number indicating higher similarity). For example, A549 had a higher expression of SLCO1B1 (solute carrier organic anion transporter family, member B1), ARNT2 (nuclear receptor), BLMH (bleomycin hydrolase) and MT1A (metallothionein 1A), while NCI-H358 did not express these genes, indicating that A549 may differ in uptake, gene induction, metabolism, and toxic stress response. We may therefore assume that A549 is an appropriate cell model for investigating the level of intracellular ROS generated by particle-bound PBDEs. However, BEAS-2B cells can be also considered as a second cell model due to the highest similarity to primary lung cells (Courcot et al. 2012).

It is important to recognize that toxicological studies at physiologically- and environmentally-relevant concentrations are essential for risk assessment of PBDEs. However, a major aspect of the work described in present study is developmental work, i.e. evaluating our *in vitro* exposure system by comparing data obtained with literature results

from traditional *in vitro* studies *via* medium exposure. Those *in vitro* toxicity studies often employed high concentrations (from 5 to 100 μM) (Barber et al. 2006; Zhang et al. 2013). The *in vitro* doses of particle-bound PBDEs used in this study ranged from 10 to 22 nmol/cm^2 (approximately equivalent to 50 - 100 μM in traditional liquid *in vitro* methods) which is in the range of PBDE exposure concentrations used in most *in vitro* studies. Despite the need of toxicological studies at environmentally and biologically relevant concentrations, little is known about such exposure levels of PBDEs for *in vitro* investigations and only a few studies (Barber et al. 2006; Li et al. 2012; Napoli et al. 2013; Wei et al. 2010) have examined the effects of PBDEs *in vitro* at lower concentrations (10 to 100 nM). In particular, a recent study provided an approximate reference for setting environmentally relevant exposure concentrations (Wei et al. 2010), suggesting approximately 10 nM as highest environmentally relevant PBDE concentration for cells (MCF-7, HepG2, H295R and PC12) in medium. However, since under these assay conditions, only 2-5% of PBDEs is expected to partition into the cells (Barber et al. 2006), the cells would be exposed to lower doses of PBDEs than intended. Thus, the environmentally relevant exposure concentration should be adjusted to approximately 200 nM for cells in medium. In addition, those cells are derived from internal organs (breast, liver, adrenal gland) which are not the first site of contact. The one-time exposure doses used in our exploratory study are relatively higher, but lower doses of PBDEs and repeated dosing mimicking chronic exposure of lung cells to air contaminants are therefore planned for our future work.

In vitro exposure systems employing an ALI to assess the toxicity of inhaled particles have received considerable attention in recent years (Kim et al. 2013; Lenz et al. 2009; Lichtveld et al. 2012; Rothen-Rutishauser et al. 2009; Volckens et al. 2009). Many of these studies have illustrated limitation and problems of *in vitro* exposure of submerged cells to particles. Results by Lichtveld et al., using a direct *in vitro* sampling and exposure method (DSEM) demonstrate that the resuspended particulate matter (PM) collected on the filter into liquid significantly modified its toxicity compared to direct deposition due to the changes in chemical composition of the PM prior to the delivery of PM to the cellular surface (Lichtveld et al. 2012). Although submerged *in vitro* testing methods for assessing particle toxicity have beneficial advantages compared to ALI *in vitro* systems, results obtained from solution cell culture methods may represent only a lower bound on PM toxicity and it should, therefore, be used with caution (Lichtveld et al. 2012).

We recognize that directly comparing *in vitro* results to *in vivo* measurements is difficult for a number of reasons, including the fact that there are over 40 cell types in the lung. Cell lines have often lost the specific functional properties of their *in vivo* equivalents and do not always provide full and stable phenotypes (Prieto et al. 2006). There are a number of differences between *in vitro* and *in vivo* studies (e.g., different species, exposure doses and times). Despite these limitations the direct comparison of *in vitro* and *in vivo* findings is informative. It is very important to select relevant endpoints in the comparisons of particle toxicity *in vitro* and *in vivo*. Since cytotoxicity is a major inducer of inflammation for particles *in vivo* and *in vitro*, cell viability indicating cytotoxicity in *in vitro* studies and neutrophil recruitment as an indicator of inflammation in animal studies were selected to assess pulmonary toxicity of particle-bound PBDEs. Our comparative *in vivo* studies showed that PBDE particles produced increased recruitment of macrophages and neutrophils and particle phagocytosis in the lungs of rats. These data demonstrate the use of our exposure model for particle-bound contaminants in a manner that represents cellular responses of the pulmonary epithelium *in vivo*. This method and these observations can be used as guidance in the development of future ALI experiments with mixed cell cultures to explore mechanisms, interaction effects and structure-activity relationships.

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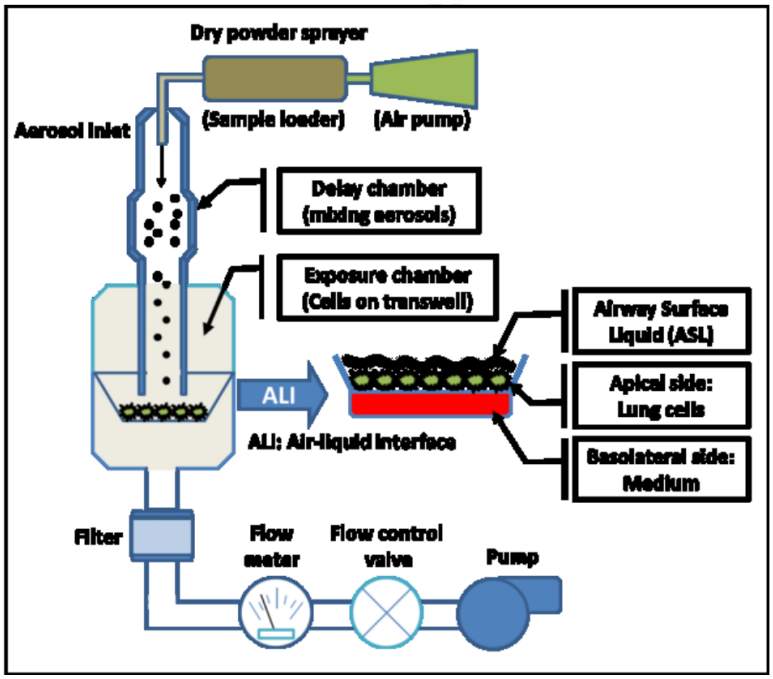
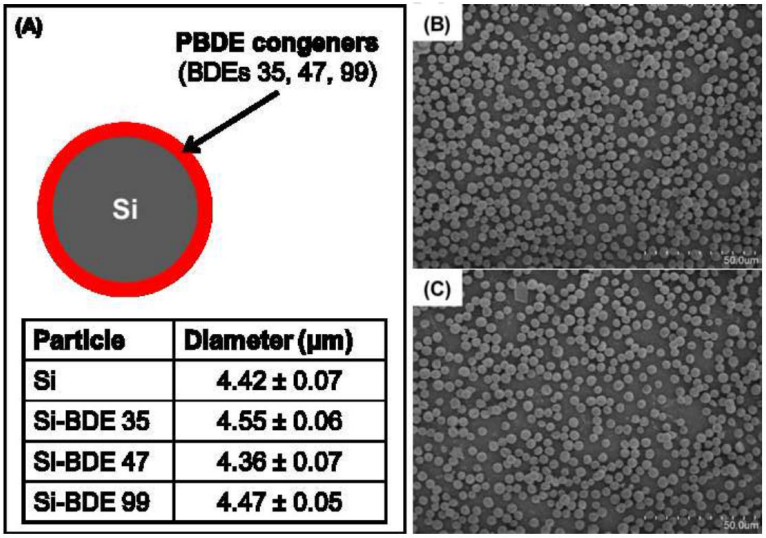


Figure 1.

Cross-sectioned representation of the *in vitro* exposure chamber. The exposure chamber delivers the dry particles to lung cells directly by low air flow (10 mL/min). The *in vitro* exposure model for particle-bound PBDEs consists of silica particles coated with individual PBDE congeners (BDEs 35, 47, and 99) and a dry powder sprayer connected to a delay chamber and an exposure chamber holding Transwell® inserts.



Particle	Diameter (μm)
Si	4.42 ± 0.07
Si-BDE 35	4.55 ± 0.06
Si-BDE 47	4.36 ± 0.07
Si-BDE 99	4.47 ± 0.05

Figure 2.

Particle-bound PBDEs were synthesized by coating silica (Si) particles with individual PBDE congeners (BDEs 35, 47 and 99). Particle size (μm) of silica and silica coated with individual PBDE congeners was analyzed by measuring the diameter of 100 random particles by scanning electron microscopy (SEM) and ImageJ software (NIH, USA) after air-delivery of particles onto the transwell in the exposure system (A). Data are expressed as means ± standard error (SE). The deposited particles on the membrane at 2 mm (B) and 2.8 mm (C) from center are shown.

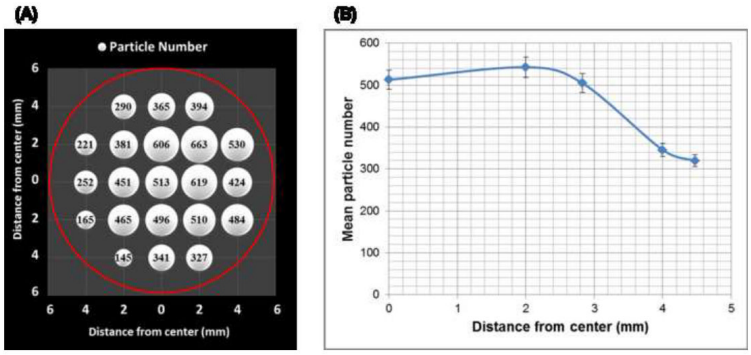


Figure 3.

The spatial uniformity of particle deposition in the system was evaluated by counting particles with a light microscope equipped with Image software (Image J) in different distances from center of transwell membrane. **(A)** The number of deposited particles on the transwell showing positions of delivered particles. The circle indicates the entire transwell (diameter, 12 mm). **(B)** The number of particles deposited on the transwell at five distances (0, 2, 2.8, 4, 4.5 mm) along the membrane radius. Data are expressed as means ± standard error (SE).

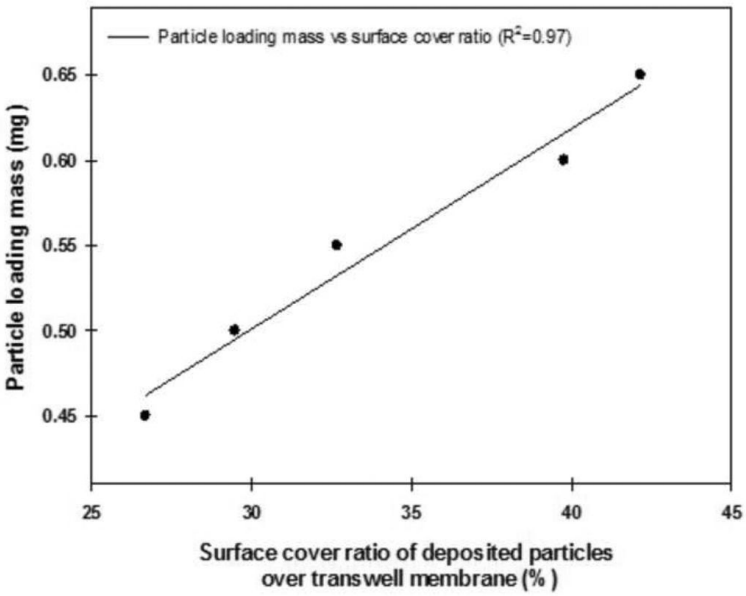


Figure 4.
Particle dosing efficiency determined by the correlation between particle loading mass and surface cover ratio.

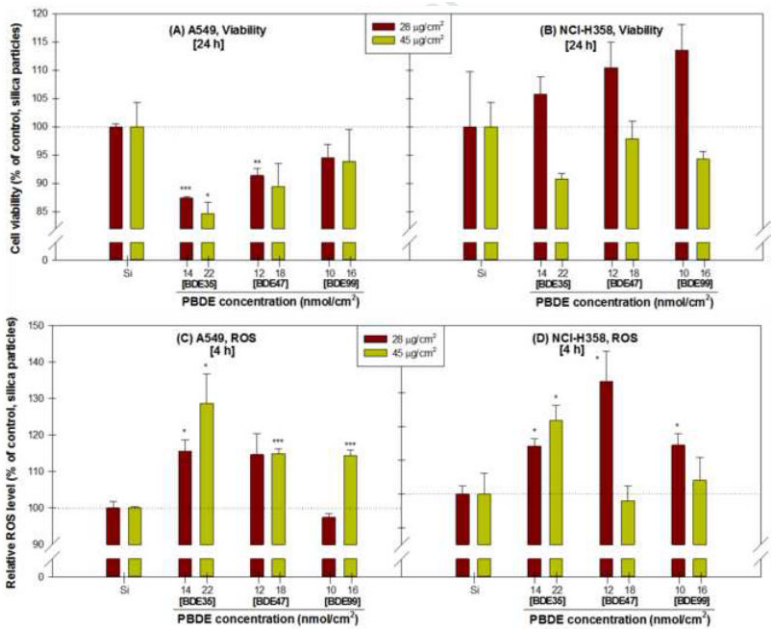


Figure 5.

Effects of particle-bound PBDEs (BDEs 35, 47 and 99) treatment on the viability at 24 h (A and B) and reactive oxygen species at 4 h (ROS, C and D) in A549 and NCI-H358 cells at an air-liquid interface, expressed as % of control. Two mass concentrations (28 and 45 μg/cm^2) particle-bound PBDEs were directly delivered to lung cells with air. Data are expressed as means ± standard error (SE). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control group.

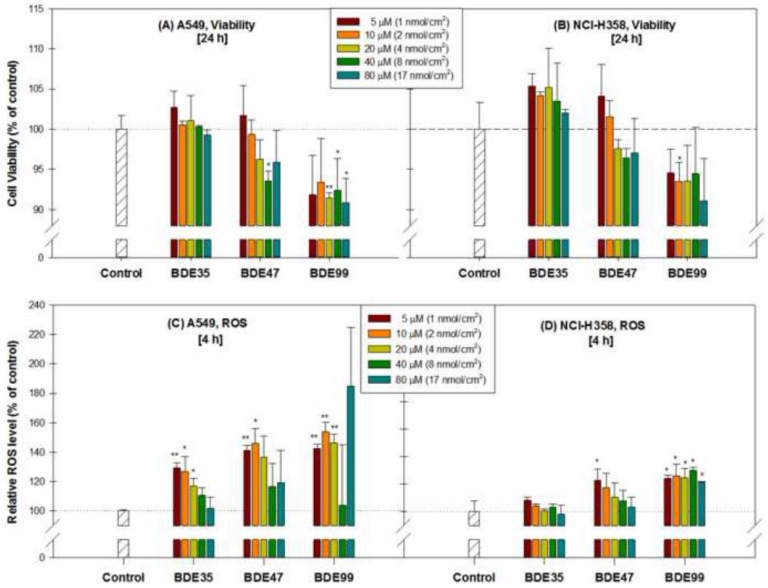


Figure 6.

Effects of PBDEs (BDEs 35, 47 and 99) dissolved in culture medium on viability at 24 h (A and B) and on intracellular reactive oxygen species formation at 4 h (C and D) in A549 and NCI-H358 cells, expressed as % of control. Data are expressed as means ± standard error (SE). * $p < 0.05$, ** $p < 0.01$, compared with the control group.

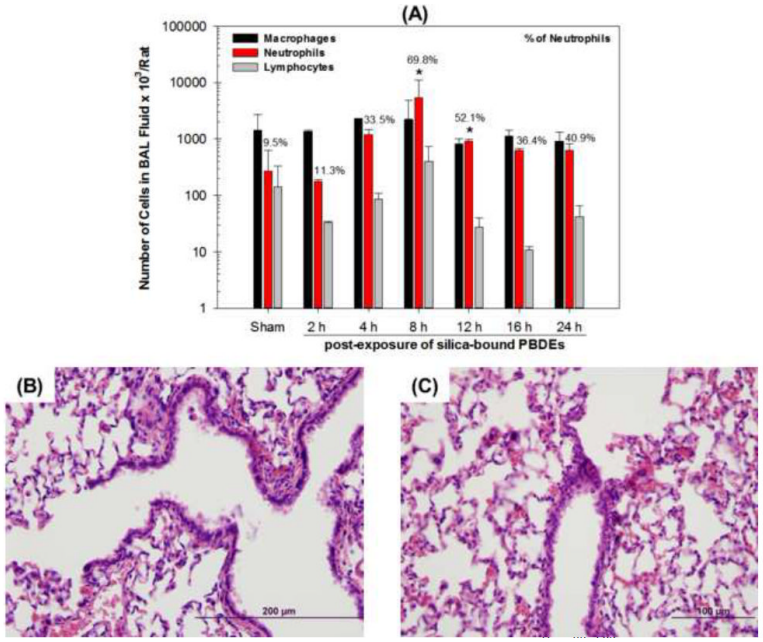


Figure 7.

Number of cells and percentage of neutrophils in BAL fluid (A) and micrographs of lung sections stained with H&E from rats exposed acutely via intratracheal insufflation of silica (B) and particle-coated with mixtures of PBDE congeners (C, 0.6 mg/rat). Data are expressed as means ± standard deviation (SD). * $p < 0.05$ significantly different from sham-exposed animals.

Table 1

The 95% confidence intervals for tine number of particles on transwell (#/cm^2) are computed based on Poisson distribution assumption.

Particle loading mass (mg)	95% confidence intervals		
	[a]Lower Bound (LB)	[b]Upper Bound (UB) Interval	Length (UB-LB)
0.45	1,731,184	1,746,725	15,541
0.50	1,913,572	1,929,911	16,338
0.55	2,120,580	2,137,777	17,196
0.60	2,581,970	2,600,940	18,971
0.65	2,737,362	2,756,893	19,531

[a]The lower bounds of the 95% confidence interval for mean

[b]The upper bounds of the 95% confidence interval for mean