Development of a dose-controlled multiculture cell exposure chamber for efficient delivery of airborne and engineered nanoparticles

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Abstract. In order to study the various health influencing parameters related to engineered nanoparticles as well as to soot emitted by Diesel engines, there is an urgent need for appropriate sampling devices and methods for cell exposure studies that simulate the respiratory system and facilitate associated biological and toxicological tests. The objective of the present work was the further advancement of a Multiculture Exposure Chamber (MEC) into a dose-controlled system for efficient delivery of nanoparticles to cells. It was validated with various types of nanoparticles (Diesel engine soot aggregates, engineered nanoparticles for various applications) and with state-of-the-art nanoparticle measurement instrumentation to assess the local deposition of nanoparticles on the cell cultures. The dose of nanoparticles to which cell cultures are being exposed was evaluated in the normal operation of the in vitro cell culture exposure chamber based on measurements of the size specific nanoparticle collection efficiency of a cell free device. The average efficiency in delivering nanoparticles in the MEC was approximately 82%. The nanoparticle deposition was demonstrated by Transmission Electron Microscopy (TEM). Analysis and design of the MEC employs Computational Fluid Dynamics (CFD) and true to geometry representations of nanoparticles with the aim to assess the uniformity of nanoparticle deposition among the culture wells. Final testing of the dose-controlled cell exposure system was performed by exposing A549 lung cell cultures to fluorescently labeled nanoparticles. Delivery of aerosolized nanoparticles was demonstrated by visualization of the nanoparticle fluorescence in the cell cultures following exposure. Also monitored was the potential of the aerosolized nanoparticles to generate reactive oxygen species (ROS) (e.g. free radicals and peroxides generation), thus expressing the oxidative stress of the cells which can cause extensive cellular damage or damage on DNA.

1. Introduction
Research on airborne nanoparticles has already proven that they can have adverse impacts on health, affecting primarily the respiratory and the cardiovascular systems [1-3]. This body of research naturally has led to increased concerns on the potential of Engineered Nanoparticles (ENP) to also cause adverse health effects and occupational safety impacts, since increasing use of ENP is caused by the growing nanotechnology market. As any adverse public or ethical reaction to nanotechnology-
based products is thus bound to create a very large economic impact, the scientific assessment of the potential health risks associated with nanotechnology-based materials and products is of utmost importance. Exposure scenarios to ENP and risk assessment at the workplace by EU organizations [4] and/or leading chemical industries [5] indicate that the potential health risk of ENP is mostly concerned with inhalation exposure of ENP dusts or aerosols (if e.g. sprayed), while dermal exposure of industrially relevant ENP formulations was found to pose essentially no risk [6]. Several studies [7, 8] point to the high importance of redox interactions between nanoparticles and cells and the relevance of oxidative stress as a very appealing paradigm for discriminating the adverse effects of different nanoparticles at the cellular and molecular level [9]. ENP made from mixed/doped oxides with varying redox activities find numerous applications in catalytic engineering, energy production, composite materials, electronic and optical devices and are largely unstudied in the context of health impacts compared with commodity ENP such as titania, silica and carbon black. Exposure to such redox ENP may eventually result in systemic inflammation and potentially promote the progression of atherosclerosis and precipitate acute cardiovascular responses as in the case of airborne incidental nanoparticles [10].

Since main contact with nanoparticles occurs during inhalation at the lung epithelium, there is an urgent need for appropriate sampling devices and methods for in vitro cell exposure studies. Traditionally, these in vitro experiments have been performed under liquid cover conditions, where nanoparticles are added to the culture medium [11]. Despite the simple and fast toxicity screening procedure that these experiments may offer, liquid cover conditions do not resemble the real exposure of the lung to airborne nanoparticles which occurs at air-liquid interface (ALI) in vivo. Moreover, the dose of nanoparticles which finally reach the cell culture remains undefined as the nanoparticles are not directly applied to the cells [12]. On the other hand, direct exposure of the cell cultures to aerosolized nanoparticles at the air-liquid interface not only represents more realistic conditions of lung exposure, but it also overcomes the uncertainty of the dose determination. The nanoparticles remain unchanged before deposition and directly contact the layer of cells which are grown on a porous membrane and are fed nutrients from below. This configuration better resembles the in vivo condition of exposure of airway epithelial cells to nanoparticles.

A variety of in-house and commercially available ALI cell culture exposure systems have been described and tested in the literature and summarized in recent papers (e.g. [13, 14]). In most of these techniques, the design is relying on diffusion and/or gravitational settling deposition mechanisms of the nanoparticles, as the aerosol flows directly to the cell culture surface by an inlet tube ending above it (basic configuration of commercial CULTEX® exposure system) using a stagnation point flow system [15]. Despite the advantageous operation compared with the liquid cover exposure, in this configuration the deposition efficiency of nanoparticles is typically low over a broad size range of 50 to 500 nm in (mobility) diameter; deposition efficiencies from 0.7% [16] to 2% [17] (for 200nm diameter particles) have been reported in the literature for systems with the aforementioned deposition mechanisms.

In MAAPHRI project [9], a specific concept for a cell culture exposure chamber had been introduced to allow the uniform exposure of cell cultures to Diesel aerosols (Figure 1a). The concept of this design is based on the stagnation point flow, but only to direct the aerosol flow through an inlet slit to be parallel to the cell culture surface, resembling in a more realistic way the in vivo lung exposure [18]. This design has been improved in an upgraded Multiculture Exposure Chamber (MEC) for in vitro continuous flow cell exposure tests (compared to the previous MAAPHRI version) regarding the geometrical arrangement of exposed cell cultures in order to increase the throughput screening possibility by 50%. Thus, the newest version of MEC can accommodate testing of up to 36 cell cultures in comparison with the 24 cell cultures in MAAPHRI concept (Figure 1b) (considering 6-well plates). The objective of the present work is the further advancement of the aforementioned MEC into a dose-controlled system for efficient delivery of nanoparticles to cells and its validation with various types of nanoparticles (Diesel engine soot aggregates, engineered nanoparticles for various
applications) with state-of-the-art nanoparticle measurement instrumentation to assess the local deposition of nanoparticles on the cell cultures.

2. Materials and methods

2.1. Exposure system with Diesel engine exhaust

In order to characterize and evaluate the Multiculture Exposure Chamber, exposure experiments of a free of cells device to Diesel engine exhaust have been performed considering the experimental setup shown below (Figure 2).

A sample from the Diesel engine exhaust (at a steady engine operation point: speed and torque, 2250 rpm and 101 Nm, respectively) is being diluted (dilution ratio 1:250) and thermally pretreated before it enters the exposure chamber. The dilution of the sample is considered as a crucial experimental factor and the final dilution ratio is selected in order to adequately simulate the “real”
exposure of human lung cells to the environmental particulate pollution [18]. The temperature of the exposure chamber is constantly kept at 37±1°C with the aid of a specially constructed oven. Soot particle concentration and size distribution in the aerosol phase from Diesel engine exhaust are being recorded “upstream” and “downstream” of the exposure chamber with a Scanning Mobility Particle Sizer (SMPS, TSI model 3936). The total particle concentration is being recorded with a Condensation Particle Counter (CPC, TSI model 3022). The diluted upstream total soot particle concentration is 2.5·10^5#/cm^3, in average. The exhaust flowrate is controlled at 2.2 L/m and the duration of the exposure experiment is 3 hours.

2.2. Flow visualization experiments
In order to assess the uniformity of the flow distribution as well as soot particle deposition, the performance of the exposure chamber was studied using Computational Fluid Dynamics (CFD) and particle transport simulation within the chamber volume in a previous study [19]. In the present study, flow visualization experiments have been performed in order to facilitate our visual perception of the aerosol velocity uniformity in the MEC by adding a tracer. More specifically, a gaseous stream of colored (blue) smoke is driven in the MEC at the flowrate 2.2 L/m. The flowrate is controlled by means of an ejector type vacuum generator in the exit of MEC (Figure 3). The distribution of the flow inside the MEC is being recorded by a digital camera through the transparent top ports of the MEC.

2.3. Control of particle deposition
During the acellular exposure experiments described in section 2.1, samples of Diesel engine soot nanoparticles are collected to be analyzed by Transmission Electron Microscopy (TEM, Jeol JEM 2010). Holey Carbon Copper TEM grids (EMS®) were placed in the 6-well plate inserts either in empty wells, or in wells containing double distilled water. In both cases, the exact position of the TEM grids resembles the level of the cell surface; proper sample holders are placed in the wells to “elevate” the TEM grids in the desirable height. The purpose of this study is to evaluate the deposition of nanoparticles on the cell surface.

Figure 3. Schematic view of the flow visualization experiment setup

In these experiments only aerosol flow distribution and velocity are being visualized and not the particle deposition in the MEC, since, firstly, the smoke particles are much larger than the nanoparticles under investigation (Diesel engine soot nanoparticles and/or ENP) and, then, the concentration is much higher than the one used in the exposure experiments.
2.4. Exposure system with nebulized nanoparticles
Characterization of the MEC for exposure experiments on cultured lung cells to aerosolized ENPs have been performed using the experimental setup shown below (Figure 4).

![Experimental Setup](image)

**Figure 4. Schematic view of the experiment setup for cell exposures to aerosolized nanoparticles**

A 100 mL solution of 20±2·10⁸ #/mL Fluoprobe532 (Interchim) labeled silica nanoparticles (Kisker Biotech) was loaded into a 1-jet Collision nebulizer (BGI Inc.) to generate the ENP aerosol. The ENP hydrodynamic diameter of 89±7 nm was determined by nanoparticle tracking analysis (NanoSight Ltd).

2.5. Cell exposure experiments
A549 cells (American Type Culture Collection, ATCC) were grown at 37°C in a 5% CO₂ atmosphere using Dulbecco’s Modified Eagle Medium supplemented with 1% penicillin / streptomycin and 10% fetal bovine serum (FBS). The cell line was maintained in 25 cm² culture flasks until confluent, rinsed once with 1×PBS, and incubated with trypsin-EDTA for 5 min at 37°C in a 5% CO₂ atmosphere. The trypsin-EDTA was then neutralized with culture medium and the cells were subsequently counted using a hemocytometer. A cell suspension with an estimated concentration of 5·10⁵ cells/mL was prepared and transwells designed to fit 6 well plates were seeded with 1 mL of cell suspension. Cells were cultured on transwells under liquid cover for the first 24 hours. Afterwards, the culture media on the apical side was removed and the cells were incubated for an additional 24 hours. One hour prior to loading in the MEC, the cells were incubated with 10 μM 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), a commonly used fluorescent probe for reactive oxygen species detection, in 1×Hank’s buffered salt solution (HBSS, Gibco). The dye solution was then removed, the well plates reconstituted with HBSS on the basolateral side of the transwells, and the well plates loaded into the MEC. The cells were exposed for 1 hour and then removed for fluorescence readings (DCF excitation/emission @ 485/530 nm and Fluoprobe532 excitation/emission @ 530/555 nm) using a microplate reader (Tecan Infinite M200). After fluorescence readings, the cells were fixed with 4% formaldehyde and processed for imaging with fluorescence microscopy (Zeiss Axiolab with Filter sets 49/DAPI, 38HE/FITC, 43HE/Rhoamine). Images were acquired with a monochrome camera (Zeiss Axiocam) and overlaid using ImageJ.
3. Results and Discussion

3.1. Collection efficiency of MEC

Diluted exhaust flow from the Diesel engine is entering into the chamber (upstream) with total soot particle concentration at 2.5·10^5#/cm³. In Figure 5 the number concentration dN/dlogDp upstream and downstream of the exposure chamber are shown as a function of the soot particle diameter, Dp.

![Figure 5. Number concentration of soot particles upstream and downstream of the exposure chamber versus soot particle diameter](image)

The average collection efficiency of the exposure chamber is defined as the ratio between the number of particles deposited in the chamber and those that are being recorded in the exit, i.e. the relative difference between upstream and downstream particle number concentrations. As a result of all repetition tests (see Table 1) the average efficiency in delivering nanoparticles in the MEC is approximately 82%.

| Test No | Total collection efficiency of the MEC |
|---------|----------------------------------------|
| No 1    | 84%                                    |
| No 2    | 79%                                    |
| No 3    | 86%                                    |
| No 4    | 80%                                    |
| Average | 82%                                    |

This high value of MEC collection efficiency is referring to the particle deposition on the whole inner surface of the chamber, at both cell growth area and “dead” surface (the stagnation plate and the margins of the insert cases). However, considering firstly that the growth area per insert is 5.71·10^{-3} m² for the case of 6-well plate inserts, then that the total growth area which can be accommodated in MEC is sixfold this surface area, and lastly that the total surface area of the exposure chamber is 8.21·10^{-2} m², then we can easily conclude that the ratio between the total growth area of MEC and the total surface area of MEC is really high (approximately 42%). Consequently, the cell-specific deposition efficiency is roughly half of the inner deposition efficiency, i.e. 35%, which is much greater than the collection efficiencies reported in the literature [16, 17]. Comparing MEC with the previous

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1 We assume that the effective design of the MEC (position of inlet ports, distances between inserts and the inlet slit etc) has minimized the particle losses in other possible “dead” surface areas.
in-house constructed exposure chamber (MAAPHRI version), the cell-specific collection efficiency of the current version has significantly increased by 120%, since in MAAPHRI version the inner deposition efficiency was 76%, but the ratio of growth area per the total surface of the chamber was only 21% (cell-specific collection efficiency 16%).

3.2. Flow and velocity uniformity in MEC

Several snapshots from the video recording during of the flow visualization test are shown in Figure 6. As $t = 0$ sec is considered the time that the flow of blue smoke/tracer is entering the exposure chamber. We notice that the flow is being distributed along the two thirds of the central inlet tube of the chamber until $t = 4$ sec and then the flow starts to be distributed along the vertical axis towards the inserts on both sides of the central tube, from $t = 4$ sec to $t = 9$ sec. At $t = 9$ sec, the flow has reached the end of the central inlet tube and starts to distribute along the most “remote” inserts from the inlet. After 34 sec, the flow has been distributed in the whole exposure chamber surface. For a typical cell exposure experiment that lasts from 1 to 24 hours, depending on the end point, this short time needed for the flow to contact each well in the chamber is not considered to cause significant effects in the uniformity and the efficiency of the exposure results.

![Figure 6. Snapshots from the video recording during flow visualization test at several intervals](image)

Nevertheless, we notice from the snapshot at the times $t = 4$ sec that a flow front starts to develop on both sides of the central tube, which is attributed to a slight non-uniformity in the flow velocity, $u$, along the central inlet tube. One way to quantify the non-uniformity is to calculate a uniformity metric $\xi$ as the ratio of the velocities between position I (at $x = 0$) and position II (at $x = L$, where $L$ is the length of the chamber in the detail in Figure 3), or to the corresponding ratio of the times needed for the blue smoke/tracer to reach the peripheral regions of the chamber (time needed from $y = 0$ to $y = \frac{1}{2}W$, where $W$ is the width of the chamber, in the detail in Figure 3):

$$\xi = \frac{u_I}{u_{II}} = \frac{\int T}{\int T} = \frac{\frac{1}{W}}{\frac{1}{L}} = \frac{1}{\frac{1}{(20 \text{sec} - 0 \text{sec})}} = \frac{1}{\frac{1}{(34 \text{sec} - 9 \text{sec})}}$$
The estimated uniformity metric $\xi$ is 1.25 for the specific geometry of the exposure chamber, revealing a slight difference in velocities along the central inlet tube which can be 25% maximum. It is believed that, in future work, this difference can be easily reduced further by manipulation of the flow field inlet through minor modification in the chamber geometry.

3.3. Soot particle deposition observation with TEM
TEM grids were placed in the 6-well plate inserts either in empty wells (Figure 3 positions B4 and E1), or in wells containing double distilled water (Figure 3 positions E3 and F2). In Figure 7, a selection of TEM pictures of the grids located both in water and in air, is shown.

![Position B4 - grid in air](image1.png)

![Position E1 - grid in air](image2.png)

![Position E3 - grid in water](image3.png)

![Position F2 - grid in water](image4.png)

Figure 7. A selection of TEM pictures from soot particles (aggregates and agglomerates) both in air and in water.

The overall picture of the TEM grids used in our experiments reveal a systematic deposition of soot particles regardless the exact position of the grid in the chamber, both in water and in the air. However, the agglomeration differs between the two cases; in the case of the empty wells, we assume that agglomerates have been formed in the wells in a greater degree due to the presence of electrostatic forces, while in water the agglomeration was attributed to the presence of van de Waals forces. In parallel, the aqueous environment has somewhat affected the external surface of the soot particles, a phenomenon which, however, is not of interest in the present study.

3.4. Cell exposure experiments
ROS production was monitored by DCFH-DA activation in A549 cells exposed to aerosolized nanoparticles for 1 hour. Comparing the fluorescence before and after exposure, elevated ROS signals were measured and visually confirmed using fluorescence microscopy (Figure 8). However, this 39%
increase in fluorescence intensity was attributed to the expected increase in DCFH-DA activation that occurs in unexposed cells loaded with the dye alone for 1 hour.

Figure 8. Microscope images of A549 cells loaded with DCFH-DA before (a & b) and after (c & d) exposure to Fluoprobe532 labeled SiO₂ nanoparticles in brightfield (left) and fluorescence (right). Magnification = 400x, Green = ROS (DCF).

Fluorescence corresponding to the Fluoprobe532 labeled silica nanoparticles appears distributed throughout the center of the transwell with aggregates visible as shown in Figure 9. Similar pictures have been taken from four more transwells in different positions in the chamber (positions A1, B5, C1, D4, E5, F3 in the detail of Figure 3).
4. Conclusions

The advancement of an upgraded Multiculture Exposure Chamber (MEC) into a dose-controlled system for efficient delivery of nanoparticles to cells and its validation with various types of nanoparticles (Diesel engine soot aggregates, SiO$_2$ nanoparticles) is presented. The average efficiency in delivering nanoparticles in the MEC is approximately 82%, while the cell-specific deposition efficiency is roughly half of the inner deposition efficiency, i.e. 35%, which is much greater than the collection efficiencies reported in the literature. A high degree of flow homogeneity has been observed with flow visualization tests, while a slight non-uniformity in the velocity (uniformity metric = 1.25) was attributed to defects in the inlet channel, which, however is easily eliminated by design modification in future work. Nanoparticle deposition was demonstrated by TEM and final validation of the dose-controlled cell exposure system was performed with exposure tests of A549 cell cultures to SiO$_2$ nanoparticles. The efficient delivery of nanoparticles as well as the homogeneity in particle deposition among the 6-well plate inserts was demonstrated by fluorescence microscopy.

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