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Nano Aerosol Chamber for In-Vitro Toxicity studies - NACIVT

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Keywords: electrostatic deposition, inhalation toxicology, air-liquid interface cultures, cystic fibrosis, cellular dose

Nanoparticles (NP) are increasingly used in industry, medicine and consumer products. Despite their great benefits, NP pose a potential risk for human health. Especially, NP in form of sprays or powders can easily be inhaled and thus the respiratory tract is the main target of undesired exposure. Individuals with pre-existing lung disease, such as cystic fibrosis (CF), are expected to be more susceptible than normal subjects. However, for efficient evaluation of adverse effect by inhaled NP, in-vitro test systems that mimic real situations are desirable. Moreover, safety testing needs to include studies in susceptible populations.

Based on previous work (Savi *et al.*, 2008; Mertes *et al.*, 2013), an improved Nano Aerosol Chamber for In-Vitro Toxicity (NACIVT, www.nacivt.ch) has been developed, which allows the deposition of aerosolized NP out of a continuous air-stream on cells cultured on Transwell® inserts.

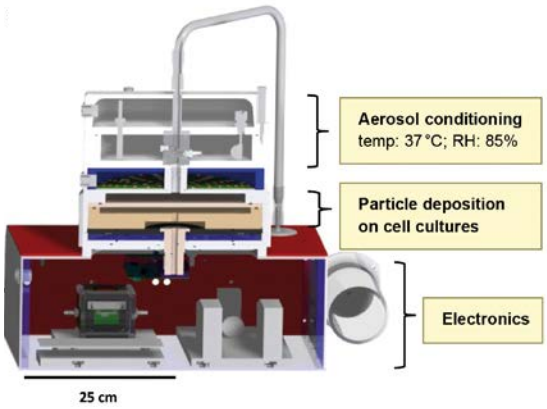

Figure 1. Design of NACIVT.

Advantages of NACIVT are:

- Compact and portable design
- Computer controlled temperature and humidity conditioning (typically 85-95% RH and 37°C)
- Integrated electrometer allowing online particle concentration measurements
- Efficient NP precipitation by electrostatic deposition
- Simultaneous exposure of 24 cell cultures, allowing high-throughput screening

NACIVT has been thoroughly characterized in terms of particle distribution and deposition efficiency, compatibility for cell exposures and particle-cell interactions.

Polystyrene-latex (PSL, 200 nm) and silver (Ag, 20 nm) particles were evenly distributed on cell-free inserts except for the edge, where fewer particles were deposited. Particle deposition efficiency was about 15% for 200-nm sized PSL particles and 40% for 20-nm sized Ag particles. The exposure treatment itself was not cytotoxic, as lactate dehydrogenase release in BEAS-2B cells exposed to inert PSL particles and particle-free air was not different from unexposed controls. Particle-cell interaction studies further confirmed the association of aerosolized PSL and Ag particles with cells.

In first exposure experiments, re-differentiated human bronchial epithelia (HBE) from normal and CF donors, cultured at a permanent air-liquid interface (ALI), were exposed to aerosols of spark-generated Ag and carbon (C) NP at three different doses.

Cellular responses were similar after exposure to both NP types, but more pronounced for AgNP than CNP. Normal and CF HBE responded differently. Exposure to AgNP resulted in a significant increase of cytotoxicity in CF compared to normal and control HBE. IL-6 and IL-8 release increased with increasing particle dose in all experimental settings. Exposure to both NP increased IL-6 secretion significantly in CF compared to normal HBE. IL-6 release in CF controls was 6 times higher than in normal controls.

This is the first study showing that subjects with a chronic airway disease respond differently to NP than normal individuals. The fully validated NACIVT combined with advanced cell models cultured at ALI has been shown to provide a realistic in vitro system for safety testing of NP.

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Utilizing cloud motion for dose-controlled, fast and efficient aerosol-to-cell delivery (ALICE technology)

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Keywords: bulk motion of aerosol, air-liquid interface cell exposure system, in vitro drug screening, nanotoxicology,

Inhalation therapy is widely used for the treatment of chronic lung diseases, but it is also attractive for non-invasive systemic drug delivery utilizing the thin air-blood barrier in the alveolar region. There is currently a surge in nanocarrier-based drug formulations, since they are able to overcome some of the limitations of conventional formulations especially regarding the use of non-water soluble and/or structurally delicate agents (e.g. proteins, peptides, anti-bodies) as well as controlled release and cell-specific targeting of the drug. As preclinical efficacy and/or toxicity testing is among the first steps in the development of new inhalation products, research in this field is expected to intensify significantly in the upcoming years. This raises the demand for efficient and dosimetrically accurate aerosol-to-cell delivery systems for liquid (and dry) substances. As most of the pre-clinical and clinical personnel are no aerosol experts, these systems should be easy to use reliably without any expert aerosol knowledge.

In this study we present the ALICE technology (ALICE: Air Liquid Interface Cell Exposure system), which is a compact, efficient, dosimetrically accurate and yet simple to operate system for delivering high doses of micron-sized droplets onto cell layers cultured at the air-liquid interface. The ALICE technology utilizes cloud (bulk) motion for rapid convective transport of a dense cloud of aerosol to the bottom of an exposure chamber, where the cells are positioned. In principle any nebulizer providing a high enough cloud density for bulk motion to occur can be used (at least 100 mg/m^3 for a cloud with 10 cm diameter; Hinds 1999). However, the diameter of the cloud, cloud density and the geometry of the chamber have to be optimized for uniform mixing of the cloud throughout the entire chamber and subsequent gravitational settling of the droplets onto the cells.

Vibrating mesh nebulizers provide sufficiently high aerosol output rates for the ALICE technology (typically >0.2ml/min) without the need for any air flow rate. Two different versions of the ALICE technology are discussed here, one using two different vibrating mesh nebulizers, an investigational eFlow (Pari, Germany) and an Aeroneb Pro nebulizer (Aerogen, Ireland) The latter system – henceforth referred to as ALICE-CLOUD - has been made commercially available by VitroCell Systems (Germany).

Both ALICE versions offer rapid delivery (<3.5-10min) of sufficiently large amounts of aerosolized liquid (ca. 1.0 µl/cm^2; <15µm thick liquid film) for drug efficacy or substance toxicity testing. The systems can hold one (Aeroneb Pro) or two (eFlow) standard multi-well cell culture plates and the well-well dose variability is <8% and the repeatability of the cell-delivered dose is <10%. Figure 1 shows the time course of aerosol deposition onto the cells in the ALICE-CLOUD equipped with an Aeroneb Pro nebulizer. Between 57 (eFlow) and 84% (Aeroneb Pro) of the nebulized drug is deposited onto the bottom of the exposure chamber, which implies that 7% (eFlow) and 17% (Aeroneb Pro) of the invested amount of liquid is deposited onto the cells for 6-well transwell inserts, with the highest cell coverage of all commercially available multi-well plate.

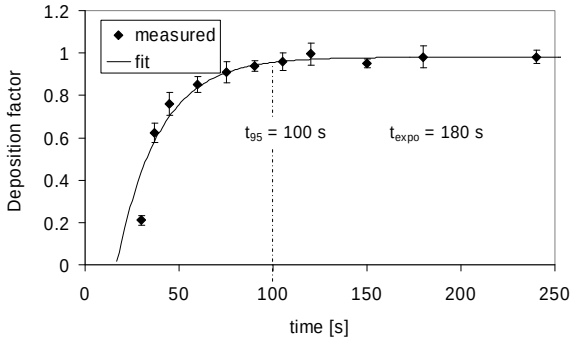

Figure 1. Time course of aerosol deposition onto cells in the ALICE-CLOUD (Aeroneb Pro nebulizer). The asymptotic deposition factor of 0.836 implies that 83.6% of the invested liquid reaches the bottom of the chamber, where the cells are positioned.

The technology has been applied to nanotoxicological (Lenz et al., 2009; Brandenberger et al., 2010) and therapeutic efficacy studies with air-liquid interface pulmonary cell systems (Lenz et al., 2009; Lenz et al., submitted manuscript).

This study was supported by the German Federal Ministry of Education and Research within the Leading-Edge Cluster “m4 – Personalized Medicine” in Munich.

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Air-liquid interface exposure system for in vitro toxicological studies of combustion derived ultrafine aerosols

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Keywords: Exposure, Health effects, Human lung cells, Dose
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Background: The inhalation of ambient aerosols can have serious impact to human health as epidemiological studies have shown. Inhalation of such aerosols can be linked to increased morbidity and mortality rates, while aerosols formed during combustion processes seem to be the most potent hazards [1]. Current attempts to replace fossil fuels by regenerative sources create new additional sources for emissions of combustion derived aerosols. As high the effort is to establish these regenerative sources, as low is the knowledge about their impact on the human being. Addressing this knowledge gap, the "Helmholtz Virtual Institute of Complex Molecular Systems in Environmental Health" (HICE) conducts joint measurement campaigns wherein controlled aerosol generation by combustion of different fossil fuels (e.g. wood, diesel) is combined with comprehensive on-line characterization of the aerosols and in vitro investigations of their effects on human cells.

Material and Methods: Most in vitro studies on aerosol health effects rely on submerged exposure of collected particulate matter, suspended in the medium. However this method does not represents the actual process in the human lung. It even changes the origin properties of the investigated aerosol. Research at the air liquid interface can eliminate these disadvantages, but requires a well engineered system to guarantee reproducible conditions. For the HICE project a new system was built based upon the Karlsruhe Exposition System [2]. It contains 18 positions to investigate aerosols as well as only the gaseous phase of an aerosol. Additionally an electrical field can be applied at every position to increase deposition efficiency. As a reference 6 positions can be used with clean air as negative control. Hence exposed cells can be compared to cells that pass the same procedure without facing the aerosol. Experiments with the fluorescein-sodium dosimetry have shown reproducible deposition efficiencies with and without electrical field. These results could be confirmed by TEM image analyses also as by numerical simulation.
Several measurement campaigns were successfully performed within the HICE project: aerosol from different biomass heaters were also investigated as the emissions of a ship diesel using 2 kinds of fuel.

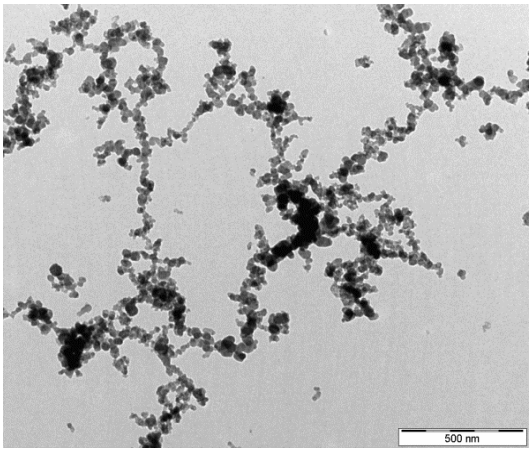

Figure 1. TEM images of diesel fuel inside the VITROCELL® Module of HICE exposure system while an exposure experiment at the ship diesel in Rostock

Results: The data are discussed with focus on the dose determination which is essential for the biological assessment via a dose-response-relationship.

Acknowledgement:
This work was supported by the Helmholtz Association within the virtual institute HICE. Further Informations: www.hice-vi.eu

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A thermal precipitator for the deposition of airborne nanoparticles onto living cells

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Keywords: nanoparticles, thermal precipitator, air-liquid interface, in vitro.

As the variety of industrial applications of nanoparticles and hence the production volume increases, concerns rise whether nanoparticles may pose risks to exposed humans, especially at workplaces (Kuhlbusch, 2011). Common methods to study hazardous effects of aerosols are in vivo studies on animals, like rats and mice. But concurrently, concerns have risen because of animal welfare and the high costs of such studies. As an alternative, in vitro studies are considered, analyzing the biological response of cell cultures after exposure to nanoparticles.

We have developed the Cyto-TP, a thermal precipitator capable of depositing airborne nanoparticles onto living cells directly from the gas (Broßell, 2013). A temperature gradient is established between two parallel plates. Therefore particles migrate toward the colder plate due to thermophoresis. The colder plate contains two transwells with attached cells forming a monolayer at the air–liquid interface. The front transwell is used for the cell exposure to particles transported into the device and deposited on the cells. Nanoparticles in the size range of 10–300 nm deposit homogeneously on the cell monolayer. All particles are deposited before the rear transwell hence its cells are exposed to the particle-free gas only, allowing for a differentiation between particle and gas effects. Figure 1 shows this concept.

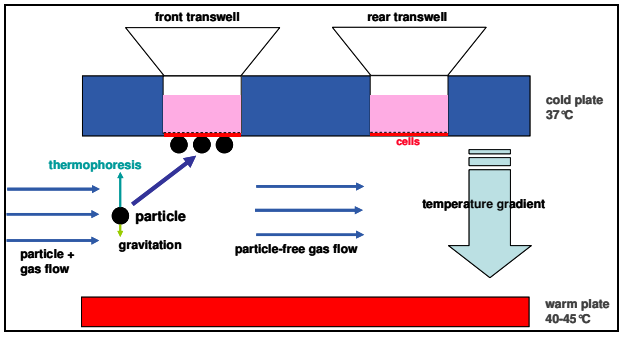

Figure 1. Concept of the Cyto-TP, a combination of a thermal precipitator and the air-liquid interface technique

The modelling of the deposition of unit-density spherical nanoparticles in the flow channel led to the design and construction of this device. The Cyto-TP was initially tested by depositing fluorescent polystyrene-latex (PSL) nanoparticles on A549 alveolar epithelial cells, a common cell line for inhalation toxicology. The predicted deposition was verified, after the absence of particles on the rear transwell after exposure and in parallel the detection of PSL particles on the front transwell. Additionally, the experimental requirements for the Cyto-TP to function have at the most an insignificant effect on the cells as proven by assaying the living cell count of the transwells after exposure to particle-free air. Consequently, potential cell damages can unambiguously be related to particle and gas exposure.

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n°211464-2.

Kuhlbusch et. al. (2011), *Particle and Fibre Toxicology*, 8, 22

D. Broßell et. al. (2013), *Journal of Aerosol Science*, 63, 75-86

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An electrostatic cross-flow precipitator for cell cultures with a highly defined deposition rate

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Keywords: air-liquid interface (ALI), electrostatic deposition, cell culture plate, defined deposition

The deposition of aerosols onto cells via an air-liquid interface (ALI) represents a realistic exposure scenario for inhalable engineered nanoparticles. However, achieving both high *and* precisely defined deposition rates remains a major challenge. We present a new type of a cross-flow ALI precipitator, in which the nano-aerosol is deposited into commercially available (in this case) 6-well cell culture plates by electrostatic precipitation. The deposition rate onto the well surface area has been measured by a specially developed technique. We present design features of the ALI precipitator, the methodology and first results.

Experimental

A schematic of the experimental setup is shown in Figure 1. As a model system for testing the device, sodium chloride aerosols in the size range of 15 to 700 nm were generated by atomization with compressed air at a flow rate of 1 l/min. The aerosol was charged positively in a self-built corona charger before being introduced into the precipitator.

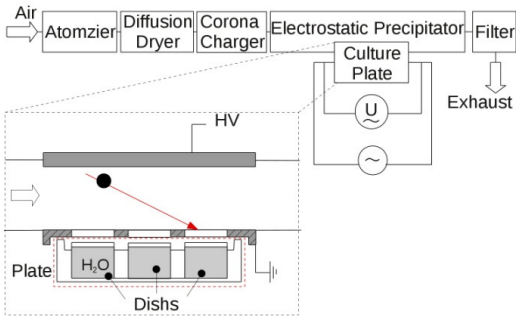

Figure 1. Setup for dosimetry measurements with sodium chloride aerosol.

At the inlet, the aerosol is distributed uniformly across the channel cross-section (dimensions 10 cm x 3 cm) and then deposited in an electrostatic field of 1 kV/cm across the well plate surface. The wells of the culture plate were each filled with DI water (conductivity of about 10 µS cm^{-1}), and the aerosol mass deposition rate was measured independently for each well by the change in conductivity of the water.

The conductivity was determined via a pair of gold plated electrodes of known cross-sectional area and spacing. With this geometry, the electrical conductivity of the solution was measured via the AC voltage and current. The accumulated particle mass per area (mg/cm^2) can then be calculated from Kohlrausch's Square Root Law, taking polarization, temperature and capacitive-resistance effects into consideration.

Figure 2 shows the precipitator, the positioning of the culture plate, and the electrode geometry.

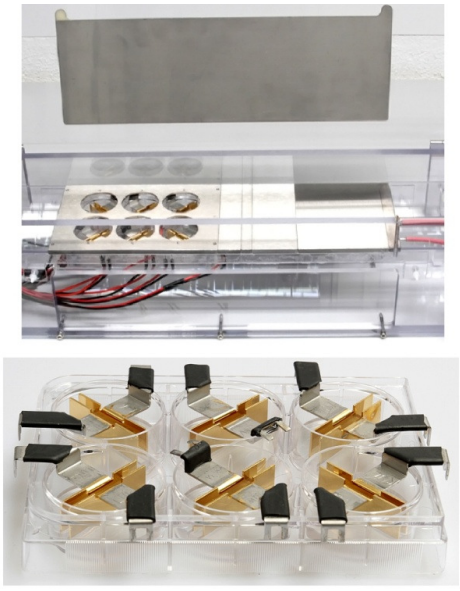

Figure 2. Top: Side view of precipitator with lid open. Bottom: 6-well culture plate equipped with conductive electrodes for on-line conductivity measurements.

First results, available at the time of writing the abstract, indicate a near-100% deposition efficiency of the aerosol entering the precipitator.

The final objective is to have a precipitator, for which the particle deposition rate can be calculated as a function of particle size and operating conditions.

Functionality Based Detection of Airborne Engineered Nanoparticles at the Workplace in Quasi Real Time

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Keywords: engineered nanoparticles, detection, workplace, catalyst, functionality, reactive oxygen species

Workers involved in the production or handling of engineered nanoparticles (ENP) are likely to be at a high exposure risk. Especially small particles can quickly attach to the background aerosol of the workplace due to coagulation. Because of the resulting disappearance in the size distribution, the existing online measurement devices are not capable to discriminate them from the background of the workplace.
Catalysis offers a good potential for the material-specific detection of catalytically active nanoparticles as well as for the discrimination of trace amounts of airborne ENP against an inactive background aerosol. We previously described a sensitive and fast measurement technique and device for the detection of a range of airborne catalyst nanoparticles in workplace air (Neubauer et al., 2011; Neubauer et al., 2013). The device, named the Catalytic Activity Aerosol Monitor (CAAM), first samples minute amounts of catalyst containing aerosol onto a filter (usually during a few minutes, depending on concentration) and then inititates a catalyst specific chemical reaction on the filter sample, which permits the quantification of an activity concentration in the air with detection limits of typically a few µg. Its metric is defined as the catalytic activity concentration (CAC) expressed per volume of sampled workplace air. We thus propose a new metric which expresses the presence of nanoparticles in terms of their functionality – in this case a functionality of potential relevance for damaging *effects* – rather than their number, surface or mass concentration in workplace air.
In this study, the CAAM was investigated towards its capability to detect traces of commonly used industrial catalysts in ambient air in quasi real time. As the influence of catalyst nanoparticles on human health and biological systems is currently not understood, we investigated correlations between catalytic and biological activity as well.

Sensitivity and linearity of the CAAM response were examined with catalytically active palladium and nickel nano-aerosols of known mass concentration and precisely adjustable primary particle size in the range of 3 to 30 nm. The smallest detectable particle mass was found to be in the range of a few micrograms, giving estimated sampling times on the order of minutes for workplace aerosol concentrations typically reported in the literature. Tests were also performed in the presence of inert background aerosols of SiO_2, TiO_2 and Al_2O_3. It was found that the active material is detectable via its catalytic activity even when the particles are attached to a non-active background aerosol.
As the ability to produce reactive oxygen species (ROS) is believed to be one of the key factors of nanotoxicology (Nel et al., 2006), the generation of ROS was measured by using the DCF method (Le Bel et al., 1992) both in a cell-free environment and with THP-1 cells (human monocytic leukaemia cells). The ability of palladium and nickel nanoparticles to produce ROS in suspension correlates strongly with the catalytic activity determined by a gas-phase reaction. This coincidence points to a link between the biological and catalytic activities of catalytically active nanoparticles. It also demonstrates the usefulness of the CAAM and its new metric for quasi-real time detection of airborne catalysts in workplace air.

Acknowledgement
The research leading to these results was funded in part by the Seventh Framework Programme of the European Commission under Grant n° 211464-2 ("NANODEVICE"). The authors gratefully acknowledge financial support from the Friedrich-und-Elisabeth-Boysen-Stiftung as well.

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Neubauer N, Seipenbusch M and Kasper G. (2013) *Annals of Occupational Hygiene 57(7): 842-852*

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Aerosol exposure chamber installation for toxicological studies of the pollutants in the workplace atmosphere

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Keywords: aerosol exposure chamber installation, aerosol dynamic channels, aerosol concentration, inhalation toxicity, carbon nanotubes

A new principle of designing aerosol exposure chamber installation (AECI) for conducting experimental studies of inhalation toxicity of different substances and materials with the purpose to substantiate their hygienic safety norms in the workplace atmosphere is discussed. AECI developed by the authors has three independent dynamic channels identical by construction but differing by the level of generated target pollutant aerosol concentrations in accordance with the tasks of the planned inhalation experiment with the use of laboratory animals.

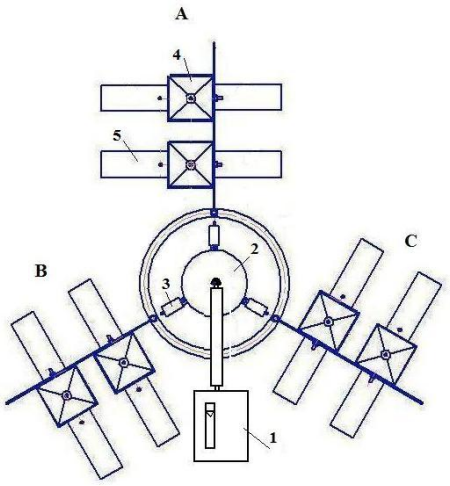

Figure 1. Conceptual top view AECI (A, B, C – AECI dynamic channels, 1- systemic aerosol generator, 2- shaper of the primary aerosol, 3- mixer of the aerodispersed flows, 4- dynamic subchannels, 5- animal restraining devices.

Aerosol concentrations of the target pollutant in each channel are adjusted within the preset limits by dosed mixing of two airflows. The first one is taken from aerodisperse flow created by general systemic generator of primary aerosol of the test pollutant; the second one is an external diluting fresh airflow. Such design allowed improving systemic technical-economical parameters of the installation compared to the already developed complex systems for toxicological assessment of the atmosphere (Salem & Katz, 2006). In particular, the target pollutant consumption in long-time experiments on chronic exposition of laboratory animals as well as the energy supply and size-weight parameters of the installation was considerably reduced. To provide statistically reliable toxicological experiments each AECI channel has 20 ports for sealed attachment of the animal (white rats) restraining devices.

Main results obtained in AECI testing with the use of aerosol of model material – multiwall carbon nanotubes (MCNT) – are given.

Table 1. MCNT aerosol concentration in the AECI dynamic channels.

Preparation	MCNT aerosol concentration, mg/m^3		
	Channel A max	Channel B med	Channel C min
0,2% MCNT suspension	10,7±0,5	4,0±1,5	1,8±0,4
MCNT powder	48,2±5,0	20,7±5,3	12,0±2,7

Salem H. & Katz S. A. (2006). *Inhalation Toxicology.* Boca Raton, London, NY: Taylor Francis Group.

Effect of zinc concentration in biomass combustion PM_1 on toxicity *in vitro*

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Keywords: biomass combustion, *in vitro*, particle formation, zinc.

Growth in the use of renewable resources in energy production has raised the interest towards health risks related to PM from biomass combustion. There is a lack of knowledge regarding the dose and composition of particulate pollutants that causes adverse effects on human health (Pope & Dockery, 2006).

Efficient biomass pellet combustion produces mostly ash in PM. The composition of the ash is mostly alkali metal sulphates and chlorides, containing also highly volatile transition metals such as Zn (Sippula et al. 2009). It has been shown that Zn concentration in biomass fuels can be one factor of PM1 toxicity (e.g. Uski et al., 2013). To study the role of zinc in metabolic activity of a macrophage cell line, the response of cells to increasing zinc concentration in biomass combustion PM was analysed.

Wood pellets doped with zinc were used as a fuel in a 25 kW pellet boiler. Pellet raw material was first milled and mixed with elemental zinc powder in various concentrations. Finally, the mixtures were pelletized for usage as fuel. Also undoped pellet, without added zinc, were included in the series. The zinc concentrations of the final particulate samples, used in the cell exposure, covered a logarithmic range of 0.1 to 250 µg/ml (Figure 1).

The particulate and gaseous emissions as well as the chemical composition and single particle morphology of the PM1 were determined. A dose-response study was carried out using mice RAW264.7 macrophage cells. Cell death, cell cycle phases and cytokine release was analysed. A sigmoid dose-response curve was fitted into the zinc concentration-ordered data using non-linear regression. From the curve, an effective dose 50% concentration (EC50) of zinc was calculated to indicate the concentration where the toxicological effect became significant.

The PM1 varied between 10 and 70 mg/MJ, and the mobility diameter of the particles between 60 and 150 nm. The morphology and composition of the particles altered due to the increasing amount of zinc. The crystallite size of the zinc oxide within the particles was found to increase as the zinc concentration of the fuel was increased.

All studied toxicological markers were affected by the addition of zinc. EC50 responsive zinc concentrations between 15 and 25 µg/ml were determined. The corresponding molarities of ZnO are approximately 180 – 310 µM. Alterations in PM properties were observed to induce variation in the toxicological profiles. This study concludes that the fine particle composition has significant effect on the toxicity of particles even in efficient biomass combustion.

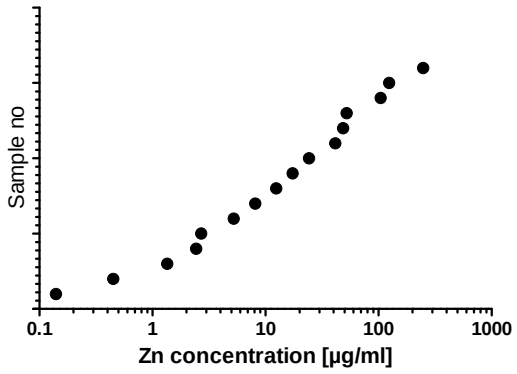

Figure 1. The range of zinc concentrations in pellet combustion PM samples used in the dose-response study. The dosing is µg to ml of cell culture medium.

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