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Laboratory-generated soot with well-defined organic coating for in vitro cytotoxicity assessment studies

Commissioned by the Federal Office for the Environment (FOEN)

Final Report

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Imprint

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1. General background and project aims

Atmospheric particulate pollution, especially soot, significantly contributes to climate change and has been linked to adverse health effects, such as respiratory and cardiovascular diseases as well as lung cancer [1,2]. These effects depend not only on physical properties of particles like concentration, size and morphology, but also on the chemical properties of airborne particulate matter (PM). Due to the complexity of atmospheric aerosols and their continuous temporal and spatial variations, it is difficult to identify, which metric(s) (e.g. particle size, number concentration, surface, mass concentration, chemical composition) dominate the onset and manifestation of detrimental health effects [3]. Specifically, (i) evidence is accumulating that metrics related to components of ambient air such as black carbon, secondary organic and inorganic aerosols may be valuable for health risk evaluation of e.g. primary combustion particles from traffic emissions, which are not fully taken into account with $PM_{2.5}$ ($\leq$ 2.5 µm diameter) mass. (ii) There are indications for a relationship between organic carbon and adverse health effects. Organic gaseous carbon emissions cause the formation of secondary organic aerosols (SOA) together with the secondary inorganic aerosols as important constituents of the $PM_{2.5}$ mass (almost 50%). Currently, discerning the toxicity of primary from secondary organic aerosols is not possible. (iii) Cardiovascular effects of $PM_{2.5}$ mass have been mainly attributed to transition metals, but no causative metal has been identified. Additional metrics beyond PM mass must be included to capture these effects. Thus, adverse health-effects studies using reference particles with well-defined coatings of either primary or secondary organic matter (but not a mixture of both as in real ambient aerosols) must be performed.

A main reason why previous research does not allow a precise differentiation of which PM constituents are most closely related to specific health outcomes is that studies were performed with real ambient aerosols, which are continuously changing. Thus, the sources and the characteristics of the aerosols are hardly controllable in such studies and they can hardly be repeated. Moreover, most are epidemiological studies, investigating the health effects of total atmospheric air pollution, including mixtures of particles and gases (e.g. NOx, O_3 etc.). This approach does not allow distinguishing the effects of specific aerosol components/ properties on health. The resulting differing and even contradictory outcomes of such studies do not allow to reliably designing new regulations. The regulations of vehicles exhaust is based on the number of particles to clearly address primary emissions and to allow the efficiency check of particle filters. For immissions, the $PM_{2.5}$ mass concentration is still a good indicator fur human health but could be complemented by minimization of carcinogenic compounds.

The aim of this pilot study (funded by BAFU) was to design and validate new experimental procedures to disentangle the effects of the different PM constituents and metrics (size, number/mass concentrations, particle surface, elemental carbon content, secondary organic matter) on cytotoxicity. This required

1) developing a stable and reproducible lab source of soot particles, with controlled core size and tunable coating of preselected semi-volatile organic matter mimicking atmospheric processes, and
2) evaluating adverse effects of these particles to the primary target tissue of inhaled particles using a realistic model system and identify particle characteristics which determine toxicity, e.g. chemical composition of coating, coating thickness, particle size, etc.

This pilot study has laid the foundation for addressing the points (i) and (ii) mentioned above with focus on ultrafine particles from traffic emissions. The effects of secondary inorganic matter and transition metals (point [iii]) as well as a more detailed evaluation of points (i) and (ii) will be addressed in a follow-up project as explained in the Section 9.

Figure 1 shows the SOA formation in the field and its translation to the well controlled laboratory model

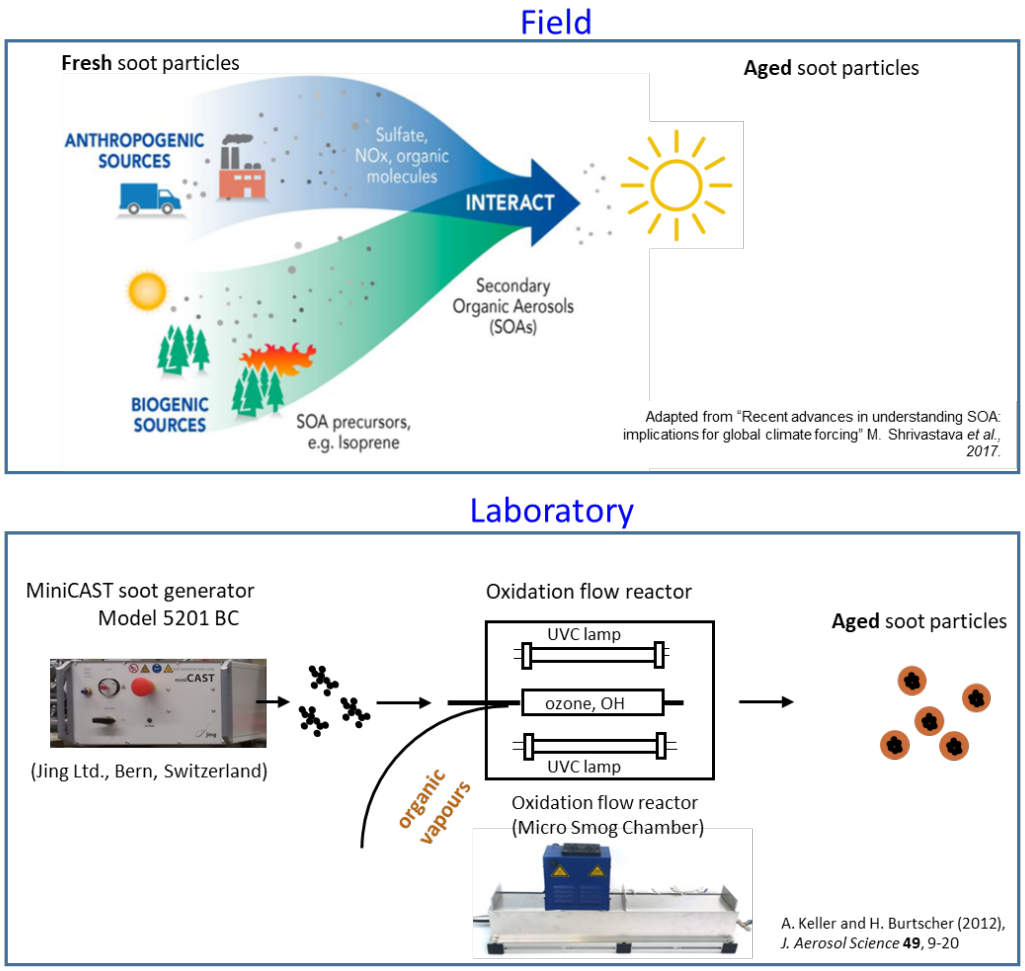

Figure 1: Schematic representation (simplified) of the SOA formation processes in the atmosphere and in the laboratory.

2. Consortium, scientific knowledge and technical developments to the interdisciplinary project

Today, significant new scientific knowledge on the broad field of air pollution and health, especially also on questions described above, can be best gained by a multidisciplinary research consortium/team consisting of research groups working at the cutting edge of scientific and technological development in the different fields. The consortium of the present study consisted of the following members bringing complementary strengths and skills to the project.

2.1. Dr. Konstantina Vasilatou and Dr. Michaela Ess, Federal Institute of Metrology (METAS), Laboratory Particles and Aerosols, CH-3003 Bern-Wabern

METAS currently participates in the European Metrology Programmes EMPIR-Black Carbon (www.empirblackcarbon.com/) and EMPIR Aeromet (http://www.aerometproject.com/). Within these two projects, the contractors of METAS have developed a new aerosol source by combining a prototype miniCAST generator [4] (Model 5201 BC, Figure 2) with an oxidation flow reactor known as micro smog chamber (developed by A. Keller at FHNW) [5]. This novel setup allows generating carbonaceous aerosols under controlled conditions.

Within the present pilot study, the collaborators at METAS have adapted the aerosol source to the needs of the project, were involved in organizing and executing the measurement campaigns as well as analyzing the experimental data recorded with an array of instruments, including a Scanning Mobility Particle Sizer (SMPS), a Tapered Element Oscillating Microbalance (TEOM), EC/OC thermo-optical analyzer and ion chromatography system.

Figure 2: Picture of the MiniCAST 5201 Type BC.

2.2. Dr. Alejandro Keller, University of Applied Sciences Northwestern Switzerland (FHNW), Institute of Aerosol and Sensor Technology, CH-5210 Windisch

The contractor of the University of Applied Sciences developed the portable photo-oxidation reactor, known as Micro Smog Chamber (MSC, Figure 3), used within this study to mimic the photochemical production of SOA in the atmosphere [5]. The Micro Smog Chamber has been successfully used to simulate the SOA formation potential of wood stove emissions [5–7] and has also been compared to other common instruments aiming to simulate atmospheric aging in the laboratory, including large-scale smog chambers [8]. More recently, the collaboration between the FHNW and METAS has led to novel uses of the MSC for standardized production of atmospheric relevant particulate matter.

The present project represents the first use of the MSC to study the health effects of atmospheric aging and particle coating. Within this pilot study, Dr. Alejandro Keller was involved in the design and setup of the experimental system, and the planning of the measurement campaigns. Furthermore, Dr. Keller supervised the functionality of the NACIVT instrument during the measurement campaigns at METAS and was partially in charge of the aerosol production and characterization using on-line measurement instruments.

Figure 3: Representation of the Micro Smog chamber (MSC).

2.3. Prof. Marianne Geiser and Dr. Zaira Leni, University of Bern, Institute of Anatomy, CH-3012 Bern

Experimental studies of adverse effects of inhaled particles in human subjects are most often not possible foremost due to ethical reasons. Inhalation studies in animals are possible, but mostly restricted to small laboratory rodents, in which the anatomy and histology of the respiratory tract significantly differs from that of the human being. In addition, the number of anthropogenic (nano)particles, which require evaluation for adverse health effects exponentially increased in recent years with the expansion of nanotechnology. Thus, the availability of adequate in-vitro models has become increasingly pressing, but extremely difficult to realize. Such model systems must mimic lung exposure with unprecedented accuracy: we need (i) an exposure chamber delivering (nano)particles as aerosols over time, in a quantitatively and qualitatively controlled and efficient way to the biological target; (ii) cell cultures replicating the morphology and function of the inner lung surface in health and disease; (iii) a set of relevant biomarkers to identify a variety of adverse effects, which are indicative for impaired lung homeostasis, i.e., initiation of pulmonary disease or interference with the course of disease; and (iv) the possibility for high-throughput toxicity screening.

In accordance with these requirements, Prof. M. Geiser and her colleague Prof. Markus Kalberer (atmospheric chemist, now at University of Basel) developed the first deposition chamber for in-vitro cytotoxicity measurements of inhaled fine and ultrafine particles [9]. An improved instrument followed [10] and in collaboration with Prof. Heinz Burtscher and his team at FHNW, the most recent instrument NACIVT (Nano Aerosol Chamber for In Vitro Toxicity) [http://www.nacivt.ch/; [11–14]] was built. The NACIVT deposition chamber, shown in Figures 4 and 5, is an all-in-one, transportable instrument, which can accommodate up to 24 cell cultures (high throughput), and internal temperature and humidity control allow long-term exposures. The performance of the chamber was characterized in dedicated experiments with realistic aerosols from various sources.

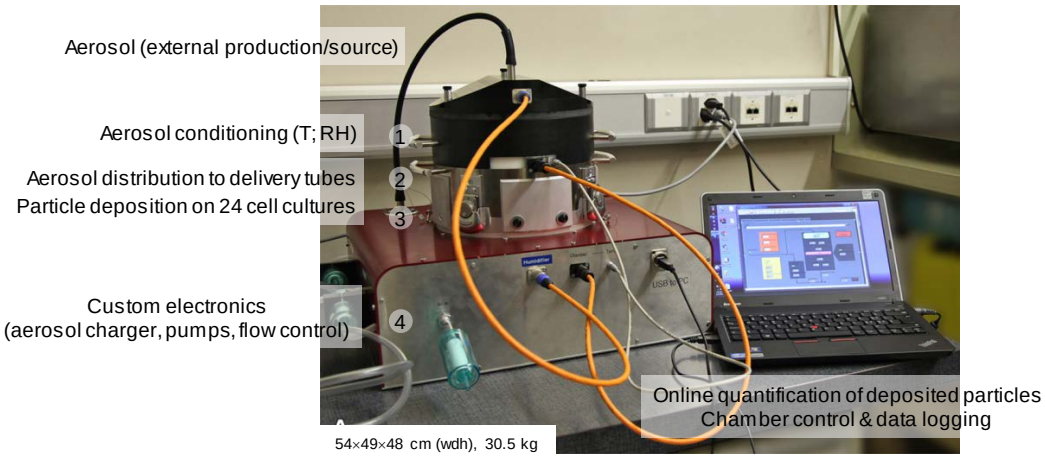

Figure 4: Nano Aerosol Chamber for In-Vitro Toxicity (NACIVT). Picture of chamber with dedicated laptop and LabVIEW software; "All-in-one", mobile system for direct use at any particle source, which mimics particle deposition in lungs (T, RH, gas, air flow, particle conc., N_{Dep}), allows simultaneous exposure of 24 cell cultures.

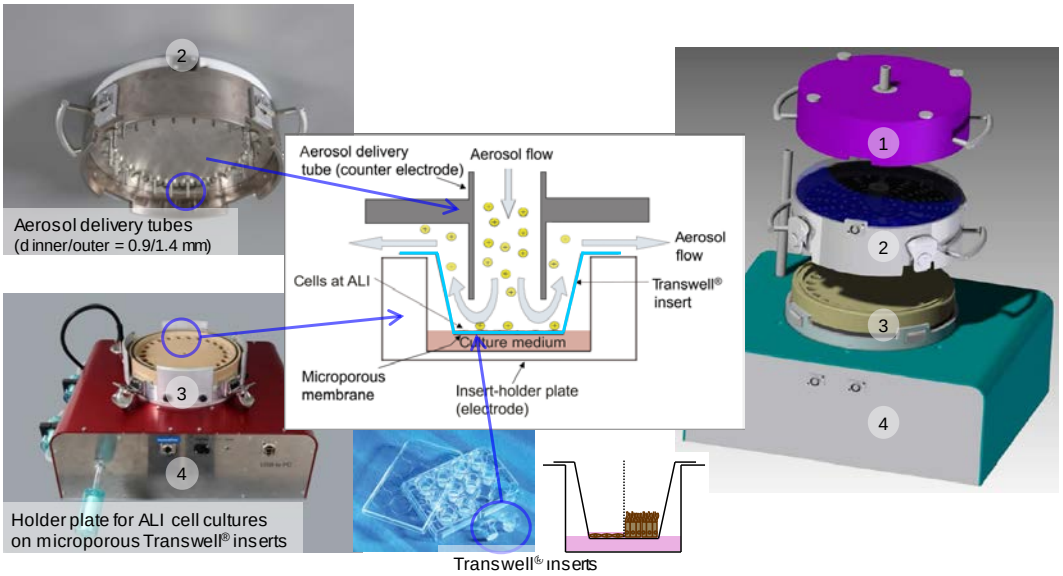

Figure 5: NACIVT – Particle delivery and deposition. Pictures and schematics showing the main parts of the chamber for particle delivery and electrostatic deposition of particles, a well-known and recognized technique in aerosol science, to reach in-vivo deposition efficiency for ultrafine particles.

Concurrently to the development of the aerosol deposition chamber, the methodology for organotypic cell cultures with lung-specific functions were established in collaboration with Prof. Matthias Salathe (pulmonologist, Universities of Miami, FL and Kansas, KS, USA) and endpoint measurements indicative for lung homeostasis selected and implemented [12,15,16]. The primary target tissue of deposited NP is the epithelium lining airways and alveoli (Figure 6).

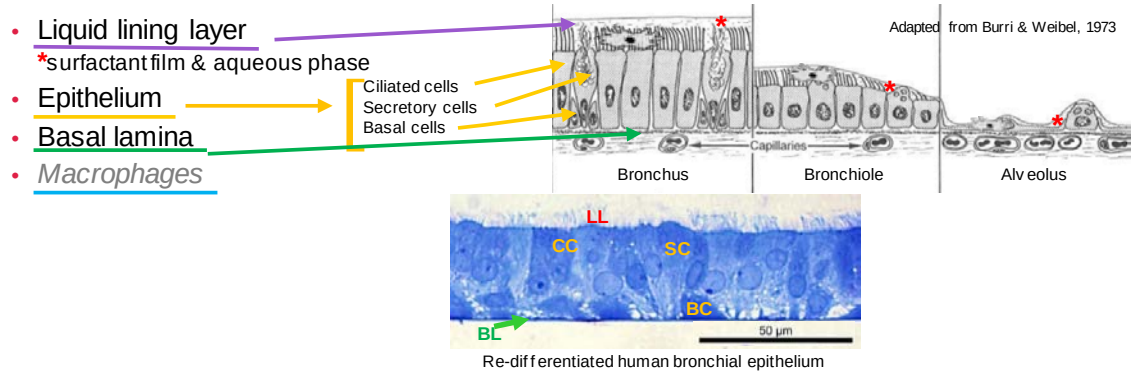

Figure 6: Re-differentiated human bronchial epithelia (HBE). **Schematic drawing** depicting the constituents of the inner lung surface (lining layer, multicellular epithelium and basal lamina), i.e. of the air conducting (bronchi and bronchioli) and the gas exchange (alveoli) compartments. Macrophages are mobile cells; their function is to clean the lung surface by uptake of deposited material or dead cells. The bronchi are the compartment of primary interest, because most lung diseases are diseases of the conducting airways. **Light microscopic image** of HBE, which closely mimics the human bronchial epithelium: all differentiated cell types, basal lamina and junctional complexes, permanent air-liquid interface, innate immunity, regeneration, long life span.

HBE cultures can be established from cells of normal human donor lungs (as shown in Figure 6) and donors with pre-existing pulmonary disease, e.g. asthma, chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF), who have been reported to be more vulnerable to adverse effects of $PM_{2.5}$. The possibility to include the susceptible population in particle-health effect studies further strengthens this in-vitro model.

To facilitate the reproducibility of exposure experiments, the contractors of University of Bern recently established a mobile cell culture laboratory, consisting of a small sterile bench and a CO_2 cell incubator, which was successfully used for experiments at the SR-Technics Zurich Airport testing facility exposing lung cell cultures to aircraft turbine engine exhaust particles [15].

Dr. Zaira Leni was responsible for the human bronchial epithelia (HBE) cell cultures, performed the cell exposures at METAS and the cell analysis at UniBern. Biological markers were analyzed at 24 h after exposure to the aerosol, reflecting an acute cellular response. We primarily assessed the induction of cell death by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) from damaged cells and the inflammatory response by measuring the release of 102 cytokines and chemokines by cells using high-throughput screening. The proteome assay technology used is a powerful tool to get information on qualitative alterations of the targeted proteins after particle exposure. In addition, we assessed cell and tissue integrity of the HBE at the ultrastructural level by transmission electron microscopy (TEM). Cilia, cellular contacts (junctional complexes), cell membranes and organelles were evaluated. TEM analysis is important to visualize the morphology of the tissue, which is crucial for its function in vivo.

3. Experimental setup

As shown in the experimental setup in Figure 7, soot particles were sampled at the outlet of a miniCAST's exhaust pipe (miniCAST 5201 Type BC) and diluted with air at a ratio of 1:10. To generate "fresh" soot particles (no coating), the aerosol was then directly delivered to a rotation disc diluter (via a bypass), where the concentration was tuned to 1.6 - 4.4·× 10^5 particles/cm^{-3}. The miniCAST 5201 BC was developed by Jing Ltd. (Zollikofen, CH) especially for METAS with the aim to generate soot particles with a high content in elemental carbon (EC). It must be noted that the EC content (expressed as EC/TC mass fraction, where TC stands for total carbon) is close to 100% for ≥ 100-nm particles but decreases to about 75% for 30-nm particles. Compared to older miniCAST models, however, the EC/TC mass fraction of the 30-nm particles has been increased by at least a factor of two. To the best of our knowledge, this is the first time it has been shown that a lab-based generator can produce carbonaceous particles with such high EC content even at small particle sizes.

After passing through a diffusion dryer and a charcoal denuder (not shown in Figure 7) to remove water and organic gas vapors, respectively, the aerosol was split and fed into a Scanning Mobility Particle Sizer (SMPS) to measure the particle size distribution, a Tapered Oscillating Microbalance (TEOM) to measure the particle mass concentration and the NACIVT chamber for cell exposure. The optical properties of the aerosol were determined with a Photoacoustic Extinctiometer (PAX, 870 nm) and an Aethalometer (AE33). To evaluate the chemical composition of the aerosols, particles were collected on quartz filters and analyzed offline with thermal-optical methods (EC/OC analysis) and ion chromatography.

To generate "aged" particles, miniCAST soot was humidified and subsequently mixed with 1,3,5-trimethylbenzene (TMB, 1st and 2nd campaign) vapors prior to oxidation in the Micro Smog Chamber. Secondary organic matter (SOM) was produced from the photo oxidation of TMB, part of which condensed on the soot particles. The thickness of the coating was controlled by varying the concentration of TMB vapors in the air stream. The physicochemical characterization of the aerosols and cell exposure in the NACIVT chamber were performed as described above for the "fresh" soot particles.

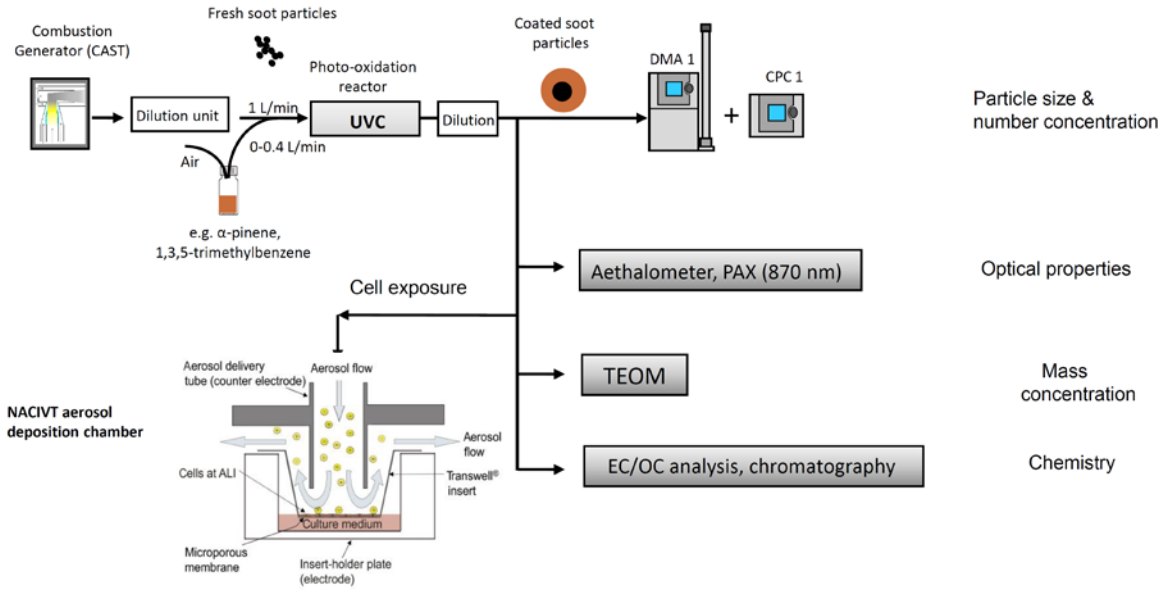

Figure 7: Schematic representation of the experimental setup at METAS for the generation and characterization of carbonaceous aerosols (fresh and aged soot particles).

The NACIVT chamber (see Figures 4 and 5 above) was not part of the original infrastructure at METAS, but could be easily integrated into the experimental setup thanks to its compact size (Figure 8).

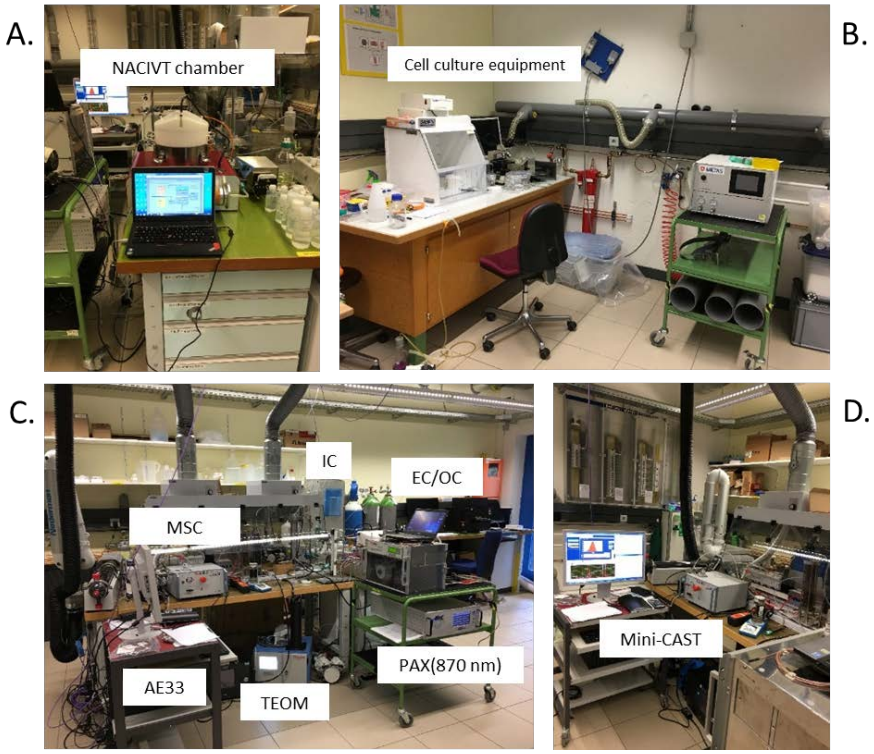

Figure 8: Experimental setup for aerosol generation and cell exposure. (A) NACIVT and dedicated computer with LabVIEW software; **(B)** Cell culture lab; **(C)** Scanning Mobility Particle Sizer Spectrometer (SMPS), Photoacoustic Extinctiometer (PAX), Tapered Oscillating Microbalance (TEOM), Aethalometer (AE33),EC/OC thermo-optical analyzer and ion chromatography system (IC); **(D)** MiniCAST.

4. Report on work performed

We developed a source for stable and reproducible “tailored” reference aerosols, which mimic atmospheric processes. Aerosols of the lab-generated soot particles were tested on the primary target tissue of inhaled particles using realistic in-vitro technology. Re-differentiated human bronchial epithelia (HBE) at permanent air-liquid interface (ALI) closely resemble the human bronchial epithelium in vivo and this cell culture model highly suitable to assess the respiratory health effects of aerosols [(18)]. The aim of this project was to combine well-controlled and chemically defined synthetic reference aerosol with state-of the-art cell analysis to distinguish the effects of specific aerosol components/properties on respiratory health.

The present report primarily describes the work within the framework of this project carried out in the period between 1. August 2018 and 31. August 2019.

Project focus:

(i) Preparation and adjustment of the NACIVT chamber for the experiments at METAS.

(ii) Performing two experimental campaigns exposing normal HBE to various soot particles with controlled coatings of oxidized TMB (13-16/08/2018; 3-17/12/2018).

(iii) Sampling of aerosol particles on TEM grids parallel to cell exposures for morphological analysis.

(iv) Analyses of biological samples from the experimental campaigns and data evaluation.

(v) Preparation of possible publication (September 2019).

4.1. Cell cultures, exposures to aerosols and sample collection

ALI cultures of fully differentiated normal HBE were exposed to the aerosols in NACIVT for 1 h. Apical washes and basolateral media were collected for biological analyses, i.e. cytotoxicity, cytokine and chemokine secretion, at 24 h post-exposure. Additionally, epithelia were prepared for (ultra)structural, i.e. microscopic analysis. Control cells were either left unexposed in the incubator (incubator control), or exposed in NACIVT to filtered, i.e. particle-free air, or to synthetic air.

4.2. Physical characterization

The particle size and mass concentrations of the generated aerosols were measured for each experiment by SMPS and TEOM. Particle mass and number concentrations, the flow rate $\dot{V}$, deposition time **T**, deposition efficiency **η**, number of wells **N** and well surface area **A** were then used to estimate the particle number (**NP**) and mass (**mP**) deposited per unit surface area on the cell culture inserts. The parameters used are shown in Table 1. The size dependent efficiency η was taken from Jeannet, et al., 2015 [20]. Tables 2 and 3 summarize the data of the physical characterization of the aerosols from all devices used. A schematic illustration of the particles used for the experiments is also provided in Figure 9.

Parameter		**Value**
ø	Insert diameter (cm)	0.65
A	Insert area (cm^2)	0.33
$\dot{V}$	Air flow (cm^3/min)	25 (for each insert, total flow =600 cm^3/min)
N	Number of inserts	24
η	Deposition efficiency	Size dependent (for results see Tables 2-4)

Table 1: Parameters used to calculate the deposited particle mass.

Test	SMPS GMD (nm)	EC/TC mass fraction (%)	Aerosol concentration		Estimated deposited particles per area within 1 h of exposure	
			Number (NP/cm^3)	Mass ($\mu g/m^3$)	Number (NP/cm^2)	Mass (ng/cm^2)
90-nm UnC	84.7	95	1.57E+05	76.0	1.88E+08	76
90-nm Max-Coat	119.0	12	1.27E+05	192.1	1.32E+08	191
30-nm UnC	34.4	76	2.81E+05	19.7	4.45E+08	28
30-nm Max-Coat	49.0	14	4.39E+05	66.5	6.15E+08	90

Table 2. Summary of particle number- and mass-concentrations: 1st campaign, TMB.

Test	SMPS GMD [nm]	Aerosol concentration		Estimated deposited particles per area	
		Number [NP/cm^3]	Mass [$\mu g/m^3$]	Number [NP/cm^2]	Mass [ng/cm^2]
90-nm UnC	94.3	1.38E+05	64.4	1.56E+08	63
90-nm Min-Coat	88.1	9.34E+04	45.4	1.37E+08	254
90-nm Med-Coat	101.2	9.58E+04	113.1	1.08E+08	70
90-nm Max-Coat	120.0	1.32E+05	265.0	1.05E+08	107
30-nm UnC	30.1	3.41E+05	3.2	5.57E+08	5
30-nm Min-Coat	36.7	2.31E+05	16.8	4.08E+08	50
30-nm Med-Coat	44.2	2.48E+05	25.4	3.57E+08	25
30-nm Max-Coat	50.2	2.90E+05	38.0	3.63E+08	35

Table 3. Summary of particle number- and mass- concentrations: 2nd campaign, TMB.

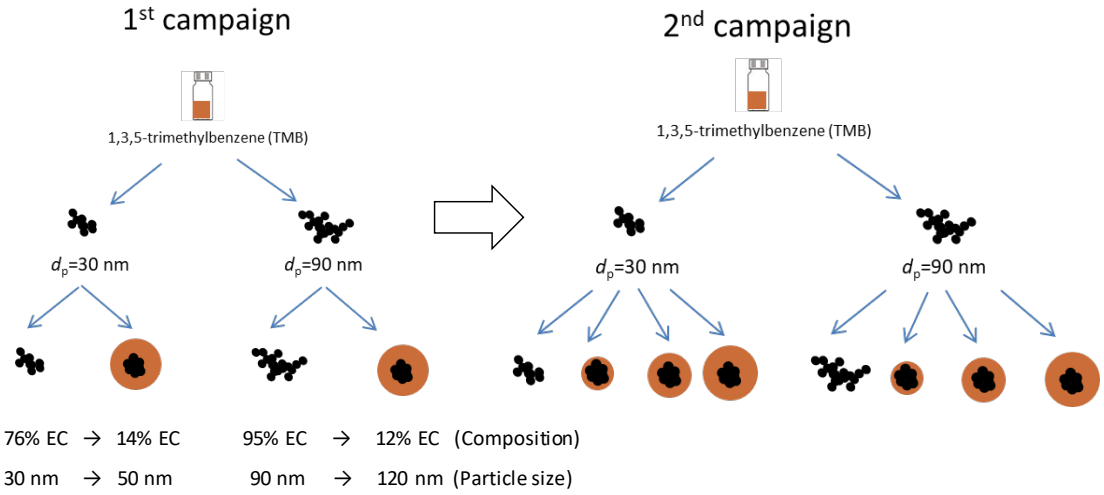

Figure 9: Schematic illustration of the particles used for the two exposure campaigns. More details on the physicochemical properties of the particles can be found in Tables 2 and 3.

4.3. Chemical characterization

First chemical analysis tests were conducted at METAS with ion chromatography coupled to electric conductivity detector and at ETH Zurich with ion chromatography coupled to mass spectrometry. The results are summarized in the following table:

published oxidation products	Found?	Chemical formula
Butanoic acid	Yes – ETHZ standard	C4H8O2
Pyruvic acid (2-oxopropanoic acid)	Yes – ETHZ standard	C3H4O3
Lactic acid	Yes – ETHZ standard	C3H6O3
Methyl maleic acid	Yes – ETHZ standard	C5H6O4
methyl maleic anhydride	Yes – ETHZ standard	C5H4O3

The above listed chemical compounds have been identified in ambient aerosol or in dedicated experiments with large-scale smog chambers. This is a first indication that the chemical composition of the coated soot aerosols presents indeed similarities with that of real ambient aerosols. More detailed chemical analysis and further validation experiments will be performed within the EMPIR project AeroTox (2019-2022).

5. Results and Discussion

5.1. Generation and physico-chemical characterization of the reference aerosols

We generated different model aerosols in each experimental campaign (see Tables 2 and 3 above), whereby two aerosols always simulated freshly emitted soot (uncoated) at nominal geometric mean mobility diameter of 30 nm and 90 nm. They therefore exhibited high EC/TC

ratio, low absorption Ångström exponent and low single scattering albedo indicating that the soot particles consisted primarily of strongly absorbing black carbon. Upon coating with SOM from the photo-oxidation of TMB, the geometric mean mobility diameter was increased in three steps to 50 nm and 120 nm. At the same time, the organic carbon content increased to about 85% for the coated 90-nm particles (typical atmospheric value of aged atmospheric aerosols). In addition, the single scattering albedo and absorption Ångström exponent increased, indicating that a coating of scattering material has been formed around the soot cores. Figure 10 shows the mobility size distributions of the uncoated soot and the size distributions of the soot, which was "aged"/coated with increasing amounts of SOM from TMB. Starting with a broad size distribution (GSD = 1.6) of the uncoated soot with nominal GMD 30 nm, the increasing amount of SOM from TMB leads to a shift of the size distribution (and thus GMD) towards larger particle sizes and a narrowing of the mobility size distribution (GSD = 1.3). This is because the SOM fills the soot voids and causes a collapse of the soot cores, leading therefore to a more uniform morphology of the particles. The same trend is observed upon coating soot with nominal GMD 90 nm.

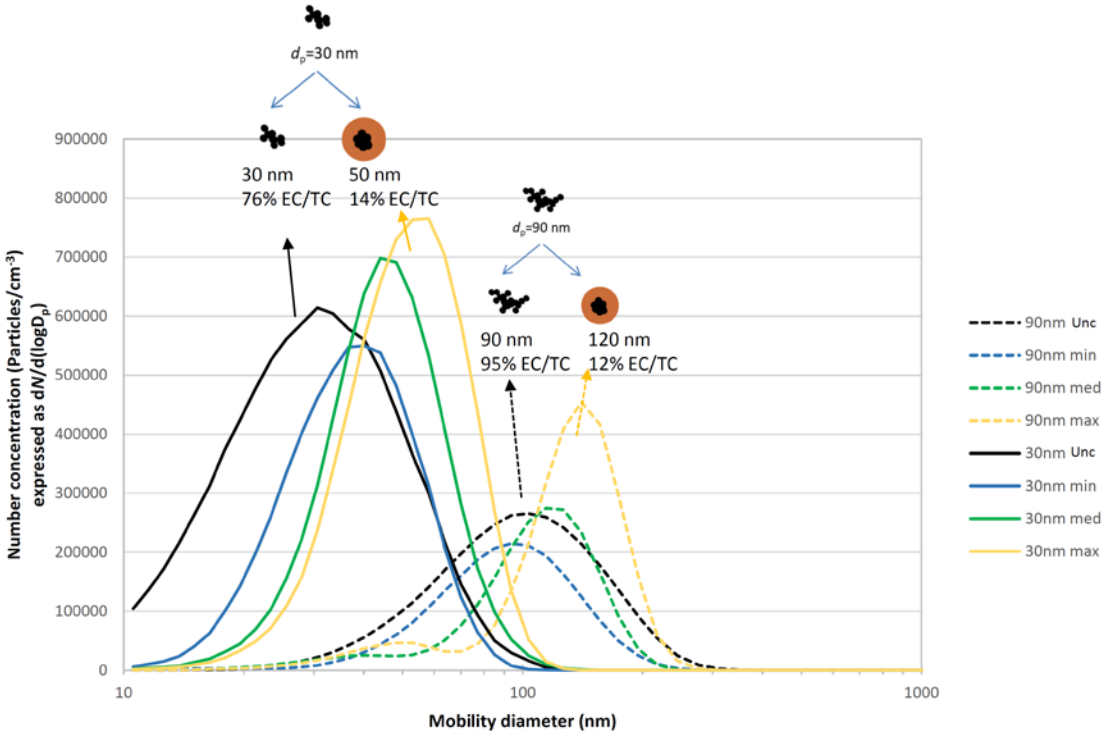

Figure 10. Mobility size distributions of uncoated and coated soot particles as measured with SMPS during the second measurement campaign. Abbreviations: Unc= uncoated soot particles, min=minimum coated particles; med=medium coated particles; max= maximum coated particles.

5.2. Induction of cell death upon exposure to aerosol

Triplicates of fully differentiated ALI cultures of normal HBE were exposed to one of the four types of soot particles. After each exposure condition, control cultures (n = 15) were exposed to the corresponding particle-free air. Synthetic air (n = 7) and untreated cells (incubator control, n = 8) served as further controls. The particle dose deposited on cells within 1 h of exposure was between 5 and 270 ng per cm^2 of cell culture area for 30-nm and 90-nm soot particles, respectively. Translating the experimentally deposited doses to real exposure conditions; they correspond to a deposited dose in the human tracheobronchial tract during

several hours in highly polluted areas (reaching $PM_{2.5}$ concentrations of 300 µg/m^3) or up to days in urban areas in Europe (with typical $PM_{2.5}$ concentrations of 20 µg/m^3) [21,22].

Biological endpoint analyses revealed particle-specific effects on HBE: Cytotoxicity, i.e. release of LDH from damaged cells was higher in HBE exposed to fresh or aged soot compared to all controls and significantly increased with increasing coating thickness (Figure 11). The highest statistically significant ($p < 0.001$) increase in LDH release was found after exposure to the 90-nm aged particles with maximum coating. This suggests that the thickness of the coating may be more important for cytotoxicity than its composition. The lowest cytotoxicity, comparable to that of control cells, was found after exposure to soot particles consisting only of elemental carbon (i.e. 90-nm uncoated soot particles (Figure 11A).

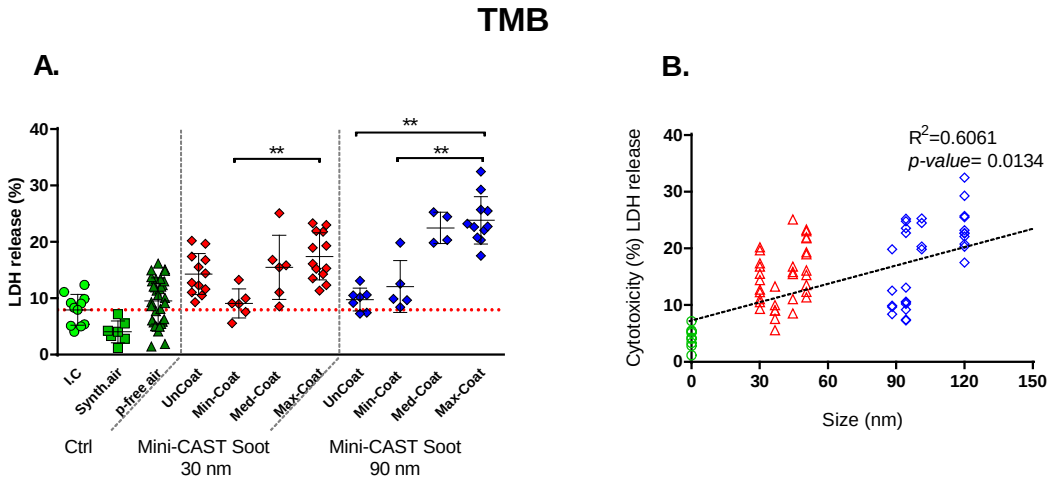

Figure 11: Cell damage in normal HBE after exposure to aerosols of fresh (uncoated) and aged (coated) particles. Cytotoxicity is presented as percentage of total releasable LDH from damaged cells into the apical compartment. Incubator control (I.C., *n*=8), particle-free air (p-free; *n*=15), synthetic air (*n*=7), 30 nm Uncoated (*n*=9), 30 nm Min-Coated (*n*=6), 30 nm Med-coated (*n*=6), 30 nm Max-Coated (*n*=9), 90 nm Uncoated (*n*=9); 90 nm Min-Coated (*n*=6), 90 nm Med-coated (*n*=6) and 90 nm Max-Coated (*n*=9). ** $p < 0.01$; using Unpaired t test with Welch's correlation.

The higher cytotoxicity of 30-nm uncoated particles can be explained as follows. Their OC/TC mass fraction is about 24%, with the OC being primary emissions from incomplete combustion. Primary OC consists mainly of polyaromatic hydrocarbons (PAHs), which are known to be toxic. Coating of the 30-nm particles with SOM decreased the LDH release to values of the incubator control. This is an indication that the SOM coating, at least in low amounts, is less cytotoxic than the primary OC from incomplete combustion.

5.3. Inflammatory response

The evaluation of the inflammatory response, i.e. the release of cytokines is important as sustained activation of pro-inflammatory mediators leads to a systemic inflammatory response, which tends to trigger cellular damage and eventually necrosis. In this study, we have semi-quantitatively measured 102 cytokines using a Proteome Profiler Human XL Cytokine Array (ARY022, R&D systems, Minneapolis, MN, USA). We found pronounced secretion of cytokines and chemokines in TMB exposed HBE compared to the unexposed controls. We then further discriminated the different types of cytokines and chemokines and their involvement in

biological processes together with the magnitude of the effect. This analysis revealed that 35 of the 102 analyzed cyto- and chemokines were deregulated in TMB exposed cells. Their classification into four group based on biological functions, i.e. (i) associated with remodeling and cell-cell interaction, (ii) implicated in cell differentiation, (iii) involved in the immune response and cell recruitment and (iv) related to apoptosis (programmed cell death), provide further evidence for substantial interference of coated TMB particles with central functions of the respiratory epithelium as a barrier including immune function. In addition, adverse effects were generally found to be more severe for 90-nm than 30-nm particles (Figure 12).

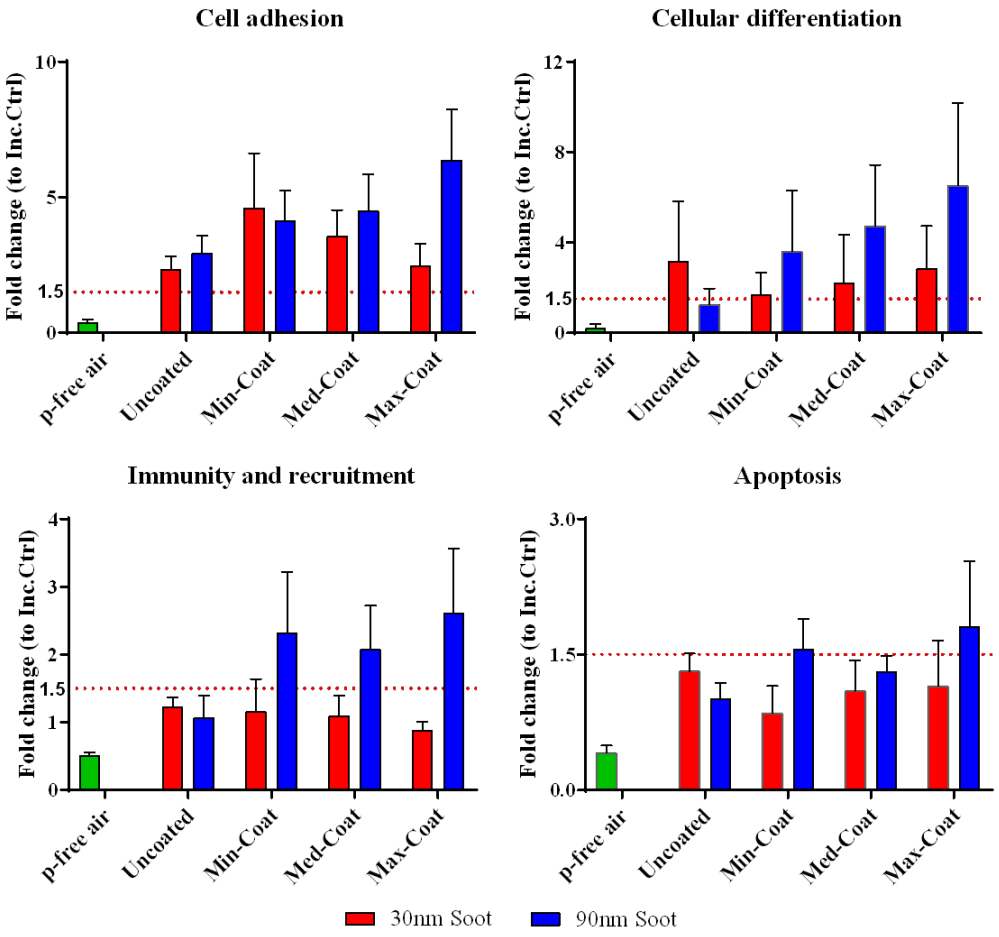

Figure 12: Cytokine array of normal HBE after exposure to aerosols of fresh (Uncoated) and aged (Coated) 30-nm and 90-nm TMB particles. Fold change relative to incubator control (Inc. Ctrl) of cytokine and chemokines related to biological pathways are reported. The data are presented as mean values and standard deviation (SD) of two independent experimental campaigns with at least triplicate cell cultures (*n* = 3 - 6 cell cultures for each condition). Dotted horizontal line: Threshold value for differential expression of cytokines and chemokines, set at ≥ 1.5 fold-change to incubator control. Abbreviations: Min-Coat = minimum coated; Med-Coat = medium coated; Max-Coat = maximum coated particles.

So far, the literature on associations between air pollution and adverse health outcomes has been dominated by epidemiological studies. However, these studies are limited in their capacity to distinguish independent effects of isolated aerosol components that cause health problems. To disentangle the adverse effects of the individual aerosol properties, there is a need for studies with well-defined reference aerosols generated in the laboratory and a precise estimation of the delivered dose. This was achieved in the present study by developing and combining techniques based on the use of "tailored" synthetic ambient aerosols, high-resolution optical imaging and state-of-the-art cell analytics. The aerosols used were to simulate the properties of real ambient aerosols whilst remaining stable and being reproducible.

The results obtained show that (i) increasing the SOM coating thickness of the soot particles progressively increases epithelial damage and (ii) indicate that soot particles coated with oxidized TMB induce stress and affect vital functions in HBE. Thereby, the response of normal HBE to TMB-soot particles exposure is mainly related to four biological pathways: Cell adhesion (e.g osteopontin, extracellular matrix metalloprotease and intracellular adhesion molecules) was the most upregulated pathway for both 30-nm and 90-nm particles (on average 3.4 fold change for 30-nm and 6.7 fold change for 90-nm particles, as compared to incubator control). This pathway consists of multi-protein complexes, which are responsible for the assembly of individual cells into the three-dimensional tissue. In line with this is the observed increase of cytokines related to the cellular differentiation (specialization) pathway, which is required to react to exogenous stimuli. Particularly upregulated were the macrophage colony-stimulating factor (GM-CSF) and the granulocyte-colony stimulating factor (G-CSF) cytokines, the overall average was a 3.3 and 5.5 fold-change increase for 30-nm and 90-nm particles respectively. Moreover, in cells exposed to 90-nm soot particles, significant upregulation of inflammatory cytokines was found, such as interleukin (IL)-6, IL-8 and IL-17A, the monocyte chemoattractant protein-1 (MCP-1) and the macrophage inflammatory protein (MIP)-3α, the overall average was a 2.6 fold-change. In addition, as a feedback loop, cells exposed to 90-nm particles exhibited upregulation of the apoptotic pathway (average fold-change 2.1). Apoptosis is a well-defined progression of genetic and morphological alterations leading to cell death. In particular, interleukin-4 (IL-4), myeloperoxidase or insulin-like growth factor binding proteins 2 and 3 (IGFBP-2 and IGFBP-3) were upregulated upon the exposure. The upregulation of these four pathways, in particular upon exposure to the 90-nm coated TMB particles, is a clear indication that HBE cells try to prevent and overcome the cell death.

6. Summary of main findings

Essential points	Results	Importance/Significance
Integrity of normal HBE after single, 1 h exposure to aerosol (LM analysis)	Lack of severe alterations examined during aerosol exposure	Cell exposures were performed under realistic conditions.
Cytotoxicity (LDH release by damaged cells)	Highest cytotoxicity after exposure to the 90-nm aged TMB particles with maximum coating.	Thickness appears to be more important than composition of coating.
OC/TC ratio	90-nm uncoated particles: contain only elemental carbon (EC). 30-nm uncoated particles: contain an OC/TC mass fraction of about 24%, with the OC being primary emissions from incomplete combustion.	Primary OC consisting mainly of PAHs reveals higher toxicity (30-nm Uncoated). Decreased of cytotoxicity after minimum SOM coating. Particles of this size with low SOM coating appear less cytotoxic than the primary OC from incomplete combustion.
Inflammation (102 cytokines and chemokines)	Deregulation of 35 cyto- and chemokines in cells exposed to oxidized TMB.	Classification into four group based on biological functions: (i) remodeling and cell-cell interaction, (ii) apoptosis, (iii) cell differentiation, (iv) immune response and cell recruitment, shows upregulation of all pathways, particularly after exposure to 90-nm coated particles. A clear indication that HBE try to prevent and overcome cell death.

Overall, with our pilot experiment using laboratory-generated soot particles we were able to identify anthropogenic precursor TMB as a harmful component of the PM. In order to protect the population and mitigate the adverse health impacts, it is important to target anthropogenic or biogenic health-relevant components.

7. Status of the activities including future steps

(i) Ultrastructural characterization of cell cultures (TEM). Ongoing
(ii) TEM-grid analysis of soot particles. Ongoing
(iii) Confocal analysis of the cell culture inserts in collaboration with National Physical Laboratory (NPL, UK, within the AeroTox project).
(iv) More detailed chemical analysis of the coated soot particles based on offline gas and ion chromatography as well as aerosol mass spectrometry (AeroTox project).

7.1 Timetable of BAFU Project 17.0094.PJ / R192-1569

Table 5: *Summary of accomplished and ongoing activities.*

Task	Month	Activities	Status
1	0 – 2	- Connecting and testing all instruments at experimental site - Generation and physico-chemical characterization of reference aerosols - Cell differentiation and phenotyping	Completed Completed Completed
2	3 - 5	- 1st series of experiments - Particle and cell analyses (1st campaign) - Compilation of results & design of further experiments	Completed Completed Completed
3	6 - 8	- 2nd series of experiments - Particle and cell analyses (2nd campaign)	Completed Completed
4	9 - 12	- Final evaluation of results - Preparation of manuscript	Ongoing Ongoing

8. Research output

Poster and oral presentations:

Leni Z. The role of laboratory-generated soot particles in respiratory health impairment. ETH Conference on Combustion-Generated Nanoparticles, ETH Zurich, June 19, 2019, Zurich, Switzerland. Oral presentation (Z. Leni).

Keller, A., Heimann, D., Specht, P., Steigmeier, P., Weingartner, E. Development of a novel portable aerosol coating unit. AeroTox Project, Stakeholder Meeting, ETH Zurich, June 20, 2019, Zurich, Switzerland. Oral Presentation (A. Keller).

Leni Z, Ess MN, Keller A, Vasilatou K, Geiser M. The role of fresh and aged soot particles in respiratory health impairment. EAC Conference 2019, Gothenburg, Sweden. Oral presentation (Z. Leni).

Leni Z, Ess MN, Keller A, Vasilatou K, Geiser M. The role of fresh and aged soot particles in respiratory health impairment. SAG Meeting 2019, Bern. Accepted for oral presentation (Z. Leni).

Publications:

"Laboratory generated coated-soot particles with tunable, well-controlled properties using a miniCAST BC and a micro smog chamber, Michaela N. Ess, Alejandro Keller, Michele Bertò, Martin Irwin, Martin Gysel-Beer and Konstantina Vasilatou, **in preparation** (METAS will acknowledge funding from EMPIR projects and A. Keller funding from BAFU).

A publication on the ***"Effects of the laboratory generated coated-soot particles on the bronchial epithelium"***, including the study with α-pinene particles ***is planned***. Main authors will be Zaira Leni, Michaela N. Ess, Konstantina Vasilatou, Alejandro Keller and Marianne Geiser; the author list will be extended depending on contributions from other collaborators. Funding from BAFU will be acknowledged; other funding sources will be added.

9. Reference to follow-up research (not financed by FOEN)

We recently performed an additional campaign with α-pinene coated soot particles, using a similar setup as for the experiments with TMB. The physical characterization of the aerosol, i.e. particle number- and mass-concentrations, is shown in the table below.

Test	*SMPS*	Aerosol concentration		Estimated deposited particles per area	
	GMD [nm]	Number [NP/cm³]	Mass [µg/m³]	Number [NP/cm²]	Mass [ng/cm²]
90nm UnC	91.4	1.29E+05	62.3	1.47E+08	62
90nm Min-Coat	88.3	1.27E+05	115.7	1.47E+08	123
90nm Med-Coat	103.6	1.32E+05	173.2	1.43E+08	179
90nm Max-Coat	121.8	1.32E+05	278.7	1.34E+08	272
30nm UnC	31.7	2.39E+05	20.0	3.85E+08	29
30nm Min-Coat	37.5	3.24E+05	26.3	4.98E+08	38
30nm Med-Coat	45.4	3.68E+05	44.3	5.34E+08	61
30nm Max-Coat	51.7	3.72E+05	65.5	5.18E+08	88

While TMB is a typical precursor molecule representing secondary organic aerosol (SOA) from anthropogenic sources, α-pinene is an important biogenic precursor. However, SOA from these two precursors have hardly been compared in realistic experimental studies with respect to their potential and mechanisms to induce adverse effects.

In view of the regulatory measures and requirements, the comparison of the results obtained with α-pinene in comparison to TMB particles will significantly strengthen the validity of the results of the present project.

The methodological developments, the experimental setup and the studies performed in this project allows closing the gap of knowledge addressed above. Further experimental campaigns including more complex aerosols and exposure of 3D biological models will be performed within the 18HLT02 EMPIR AeroTox project (2019-2022) funded by the EU.

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