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Online monitoring of volatile organic compounds emitted from human bronchial epithelial cells as markers for oxidative stress

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Abstract

Particulate air pollution is associated with adverse respiratory effects and is a major factor for premature deaths. *In-vitro* assays are commonly used for investigating the direct cytotoxicity and inflammatory impacts due to particulate matter (PM) exposure. However, biological tests are often labor-intensive, destructive and limited to endpoints measured offline at single time points, making it impossible to observe the progression of cell response upon exposure. Here we explored the potential of a high-resolution proton transfer reaction mass spectrometer (PTR-MS) to detect the volatile organic compounds (VOCs) emitted by human bronchial epithelial cells (BEAS-2B) upon exposure to PM. Cells were exposed to single components (1,4-naphthoquinone and Cu(II)) known to induce oxidative stress. We also tested filter extracts of aerosols generated in a smog chamber, including fresh and aged wood burning emissions, as well as α -pinene secondary organic aerosol (SOA). We found that 1,4-naphthoquinone was rapidly internalized by the cells. Exposing cells to each of these samples induced the emission of VOCs, which we tentatively assigned to acetonitrile, benzaldehyde and dimethylbenzaldehyde, respectively. Emission rates upon exposure to fresh and aged OA from α -pinene oxidation and from biomass burning significantly exceeded those observed after exposure to similar doses of Cu(II), a proxy for transition metals with high oxidative potential. Emission rates of biomarkers from cell exposure to α -pinene SOA exhibited a statistically significant, but weak dose dependence. The emission rates of benzaldehyde scaled with cell death, estimated by measuring the apical release of cytosolic lactate dehydrogenase. Particle mass doses delivered to the BEAS-2B cells match those deposited in the human tracheobronchial tract after several hours of inhalation at elevated ambient air pollution. The results presented here show that our method has the potential to determine biomarkers of PM induced pulmonary damage in toxicological and epidemiological research on air pollution.

1. Introduction

An increasing number of epidemiological studies have correlated elevated PM concentrations with increased mortality and various diseases such as lung cancer, asthma, chronic obstructive pulmonary disease, arrhythmia, and ischemic heart disease [1–6]. Air pollution is reported as the fifth-ranking mortality risk factor by the Global Burden Disease study (GBD) [7]. Ambient particulate matter PM_{2.5} (particle size smaller than 2.5 μ m) was attributed

to 412 000 premature deaths in Europe [8] and 4.2 million deaths worldwide [9]. Oxidative stress, induced by transition metals and organic compounds (e.g. quinones or organic peroxides) present in PM, is thought to be the main mechanism leading to inflammation and toxicity [10, 11] and may indeed be a better measure for acute health effects than PM_{2.5} itself [12]. Most epidemiological studies are based on the measurement of major health outcomes such as emergency department visits [13], cardiovascular and respiratory diseases [6, 14], and mortality [15].

However, there is lack of detailed information on the cellular/molecular mechanisms behind the observed outcomes and of clear links between exposure to specific sources and adverse health effects. In order to better understand the influence of various PM parameters (e.g. composition and sources) on biological or physiological responses, recent cohort studies, e.g. the RAPTES cohort [16] or the ESCAPE project [17], measured specific biomarkers of inflammation or oxidative stress. At present, only a limited number of biomarkers are commonly assessed/evaluated in research, like the release of cytokines, e.g. interleukin-6 (IL-6), interleukin-8 (IL-8) and the tumor necrosis factor- α (TNF α) in blood samples [18], nasal lavage [19] or supernatants of cell cultures [20, 21], or exhaled nitric oxide (NO) [18, 19, 22, 23] and biomarkers of oxidative stress (i.e. exhaled malondialdehyde and oxidized low density lipoprotein) [24]. There is thus a major need to develop innovative methods to identify biomarkers that are correlated to specific PM sources or components, in order to improve the interpretation of epidemiological studies.

Exhaled volatile organic compound (VOC) analysis by gas chromatography-mass spectrometry (GC-MS) or proton transfer reaction—mass spectrometry (PTR-MS) has been developed as a diagnostic tool more than 10 years ago to discriminate healthy from diseased individuals [25–27]. The analysis of human breath seems to be a useful routine technique to identify disease-related biomarkers, and it might be applicable more frequently to prospective cohort studies and also to cellular assays, as it is a non-invasive, fast and quantitative method. However, the precise identification of biomarkers released by pulmonary cells related to exposure to pollution requires more development and must first pass through multiple research phases to derive robust conclusions on the accuracy of the tests using these biomarkers.

In this work, using PTR-MS, we measured VOCs emitted from human bronchial epithelial cells upon oxidative stress induction by exposure to different PM components and filter extracts of aerosols. We aimed at identifying specific VOCs as biomarkers of pulmonary oxidative stress. We selected H₂O₂ as a model for peroxide compounds which may constitute a large fraction of the OA [28, 29]. Cu(II) and 1,4-NQ were chosen as representatives of inorganic and organic components of PM that induce redox cycling in the epithelial lung lining fluid or inside cells in the presence of reductants (e.g. glutathione, ascorbic acid, nicotinamide-adenine dinucleotide phosphate) [30, 31]. Since both, H₂O₂ and Cu(II) + 1,4-NQ, induce oxidative stress, released VOCs are expected to be markers of oxidative stress. We also tested filter extracts of fresh and aged OAs generated in a smog chamber from wood burning emissions and α -pinene oxidation. The emission rates of biomarkers

determined with PTR-MS were compared to the measured cytotoxicity.

2. Material and methods:

2.1. Experimental setup

Cell exposure experiments were performed with BEAS-2B cells cultured on microporous filter inserts and with minimal apical volume of Dulbecco phosphate-buffered saline solution (DPBS, Gibco, Life Technologies, Zug, Switzerland) to mimic air liquid interface (ALI) conditions. Twelve cell cultures were placed into the cell exposure chamber and flushed with purified air. The air exiting the exposure chamber was measured by a PTR-MS instrument. When a stable background level of mass signals was reached, the test sample was injected on top of each cell culture and the evolving VOCs were monitored. Cells were exposed to three types of samples (see table 1): (i) the controls using ultrapure water (negative control) or H₂O₂ (positive control for reactive oxygen species (ROS)), (ii) model compounds for aerosol exposure including CuCl₂ (CuII) and/or 1,4-naphthoquinone (1,4-NQ) and (iii) filter extracts of PM collected from either wood burning exhaust oxidation or α -pinene ozonolysis experiments. Two doses of fresh α -pinene SOA as well as extracts from 24 h-aged filters were tested. A comparison between fresh and 24 h-aged filters was performed, because Krapf *et al* [32] have shown that ROS rapidly degrade in the particle phase and a substantial fraction disappears after a few hours of aging. Measurements without cell cultures, using wells filled with DPBS exposed to particle components, served as acellular controls. Negative cellular controls were used to confirm that emissions are induced by PM components, whereas acellular controls served to verify whether the observed emissions result from the PM components themselves or from their impact on the cells. Experiments were performed 3–4 times.

2.2. Chemicals and sample preparation

1,4-naphthoquinone (purity 97%), H₂O₂ (30 wt% in water) and copper chloride (purity $\geq 99.995\%$) were purchased from Sigma-Aldrich (Sigma-Aldrich, Buchs, Switzerland). For injection onto the cells, we prepared diluted aqueous solutions of 1,4-naphthoquinone, H₂O₂, and CuCl₂. Their concentrations were adjusted such that the doses given in table 1 could be deposited with 50 μ l solution on the cells.

OA from α -pinene was produced in a 27 m³ smog chamber installed at the Paul Scherrer Institute and has been described elsewhere [32, 33]. α -Pinene OA was formed by mixing 500 ppbv of O₃ with 100 ppbv α -pinene, added volumetrically, at 50% relative humidity (RH). The particle size distribution was monitored via a scanning mobility

Table 1. List of substances and doses applied to BEAS-2B cells and acellular control (DPBS), as well as number of cell exposure experiments with 12 cell cultures each.

Test samples	Condition	Dose ($\mu\text{g cm}^{-2}$)	Number of experiments
H ₂ O	DPBS	NA	4
	BEAS-2B	NA	3
H ₂ O ₂	DPBS	100	4
	BEAS-2B	100	3
Cu(II)	DPBS	0.2	4
	BEAS-2B	0.2	3
1,4-NQ	DPBS	2	4
	BEAS-2B	2	3
Cu(II) + 1,4-NQ	DPBS	0.2 + 2	4
	BEAS-2B	0.2 + 2	3
Wood burning exhaust fresh	DPBS	0.1	3
	BEAS-2B	0.1	3
Wood burning exhaust aged	DPBS	0.1	3
	BEAS-2B	0.1	3
α -pinene SOA fresh	DPBS	0.01	4
	BEAS-2B	0.01	3
α -pinene SOA fresh	DPBS	0.1	4
	BEAS-2B	0.1	3
α -pinene SOA 24 h-aged	DPBS	0.01	4
	BEAS-2B	0.01	3
α -pinene SOA 24 h-aged	DPBS	0.1	4
	BEAS-2B	0.1	3

particle sizer (SMPS; TSI model 3938). SOA was collected on quartz filters 90 min after α -pinene injection into the smog chamber at a mass loading of 100–150 $\mu\text{g m}^{-3}$. The α -pinene SOA was passed through a charcoal denuder to remove the gas phase species prior to loading the filter at a flow rate of 20 l min⁻¹. Some filters were immediately extracted in water and used as sample in a cell experiment (fresh SOA), while the others were allowed to sit at room temperature (20 °C) for ~24 h before extraction (aged SOA).

Wood burning exhaust was investigated as well, either directly after emission (WB-fresh) or after oxidative aging with OH radicals (WB-aged) of emissions in an atmospheric chamber. Samples were collected on quartz fiber filters (20 min at 20 l min⁻¹) [34]. Emissions originated from beech wood combustion in old and modern logwood stoves. Depending on the mass loading of the filters, the diameter of filter punches and the volume of ultrapure water were adjusted to obtain the targeted OA mass for cell exposure. The filter extraction was carried out for 15 min in a warm (30 °C) water bath, then homogenized by vortexing for 1 min and filtered through a 0.45 μm nylon membrane syringe filter (Infochroma AG, Zug, Switzerland).

2.3. Cell culture

BEAS-2B cells derived from human bronchial epithelial cells (ATCC, LGC Standards sàrl, Molsheim, France) were cultured on 10 mm petri dishes (Falcon, Becton Dickinson AG, Milian, Vernier/Geneva, Switzerland) in complete medium DMEM-L (low glucose) supplemented with 10% fetal calf

serum (FCS, LabForce, Switzerland), 100 U ml⁻¹ penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin (Gibco, Life Technologies, Zug, Switzerland). Two days prior to exposure, cells were seeded at a density of 5×10^5 cells per insert on collagen I (Sigma-Aldrich, Buchs, Switzerland) coated, microporous 24 mm Transwell inserts (polyester membrane, 0.4 μm pores; Corning, Fisher Scientific, Reinach, Switzerland).

2.4. Cell exposure chamber

The home-built cell exposure chamber is described in detail by Mertes *et al* [35]. The chamber consists of three subunits: a water bath, a sample distributor and a cell exposure unit. The air flow (0.35 l min⁻¹ zero air with 5% CO₂) was conditioned to 37 °C and 85%–90% RH by the water bath and then divided into 12 delivery tubes. At the exit of the tubes, the air passed over the cells grown on inserts placed in a 12-well holder plate. A silicone rubber thermofoil below the cell chamber unit maintained a stable temperature. A polystyrene housing (4 cm thickness) further insulated the cell exposure chamber. As a new feature, an injection system consisting of a tuberculin syringe (1 ml, BD Medical, Milian SA, Vernier/Geneva, Switzerland) and a steel needle (50 $\times$ 0.8 mm, Milian) inserted into a Teflon tube (40 $\times$ 1 mm) was attached to each of the 12 delivery tubes. These Teflon tubes end a few mm above the cell culture surface.

2.5. Cell exposure experiment

Immediately before exposure, the cell culture medium was replaced by rinsing 12 cell cultures twice with DPBS and placed into the well-holder plate. Here, the apical volume was reduced to 200 μl

(approx. 0.5 mm in height) to mimic air-liquid interface (ALI) conditions. The basal volume was 2 ml. After installation of the plate inside the cell exposure chamber, we flushed the chamber with a high flow of 1.2 l min^{-1} of zero air for 10 min to remove any VOC contamination from the laboratory. After at least 50 min of baseline measurements at normal flow rate, $12 \times 50 \mu\text{l}$ ($50 \mu\text{l}$ per insert) of ultrapure water (control) or test compound solution was injected with the syringes and PTR-MS measurements were continued for two hours. Each exposure experiment was repeated at least three times. For the acellular control, plates were filled with 2.2 ml of DPBS without cell inserts and sample injection was performed as described above.

2.6. Cytotoxicity

The induction of cell death was estimated by measuring the release of cytosolic lactate dehydrogenase (LDH) from damaged cells into the apical compartment using the colorimetric LDH cytotoxicity detection kit^{PLUS} (Roche Diagnostics AG, Rotkreuz, Switzerland) according to the manufacturer's instructions. Briefly, the apical cell surface was washed once with $500 \mu\text{l}$ of DPBS for 10 min at 37°C . The apical washes were collected 4 h and 24 h post exposure and stored at 4°C until analysis. The maximum release of LDH was estimated in the supernatants of cells lysed with $100 \mu\text{l}$ 1% Triton-X solution for 10 min at 37°C . Prior to toxicity calculations, absorption values were adjusted for volume differences. Cytotoxicity is presented as percentage of maximal LDH release (absorbance) in untreated cell cultures (incubator controls). Data are expressed as mean values and standard deviation (SD) of three independent experiments with two to three triplicate cell cultures (total $n = 6$ or 9 cell cultures).

2.7. PTR-ToF-MS

A PTR-MS 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) was connected to the cell exposure chamber via a passivated stainless steel capillary (surface coating: Sulfinert[®], length: 110 cm, inner diameter: 0.76 mm, temperature: 65°C) with an inlet flow rate of 0.3 l min^{-1} . The drift tube was operated at 2.25 mbar and 115 Td ($1 \text{ Td} = 10^{-17} \text{ V cm}^2$), whereby it was kept at 65°C to prevent water condensation. The m/z 5–400 range was scanned with a signal integration time of 5 s. The PTR-TOF 8000 instrument was calibrated for acetonitrile using a dynamically diluted VOC standard (Apel Riemer Environmental Inc., Miami, USA). All other signals are reported in acetone-equivalents, i.e. the response factor of acetone ($25.1 \text{ ncps ppbv}^{-1}$) was used for converting normalized count rates (ncps) into volume mixing ratios (ppbv, by volume).

PTR-ToF-MS data were recorded using the TOF-DAQ v1.83 data acquisition software (Tofwerk AG,

Thun, Switzerland). The PTRTOF Data Analyzer software was used for data analysis [36]. Only signals showing substantial increases in the BEAS-2B experiments compared to acellular experiments were further analyzed. Peak intensity data were obtained by subtracting the baseline signal averaged for 10 min before sample injection.

2.8. UV-VIS measurement

In order to follow the internalization of 1,4-naphthoquinone (1,4-NQ) in the cells, apical and basal DPBS were collected at 5, 10, 20, 30, 40, 50, 60 min after the injection of $50 \mu\text{l}$ of 1,4-NQ onto the cells (final concentration: $2 \mu\text{g cm}^{-2}$). DPBS collected from 3 cell cultures were pooled (apical and basal separately) and centrifuged for 10 min at 1200 rpm. The absorbance of the supernatant was then measured over the wavelength range of 240–600 nm using an Ocean Optics spectrometer consisting of a 200 Hz pulsed xenon light source (Ocean Optics PX-2), a USB2000 + spectrometer, a 180–870 nm optic fiber and OceanView software (Ocean Optics, Dunedin, USA). The concentration for each data point was obtained from the absorbance at $\lambda = 330 \text{ nm}$ using a 5-point calibration curve of 1,4-NQ in DPBS. Three independent experiments for 1,4-NQ content in the apical and basal DPBS were performed.

2.9. Data analysis

Results are presented as mean values $\pm$ SD. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc. San Diego, USA). Mean values and linear regression were compared using the unpaired Student's *t*-test with ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$. The data passed the Shapiro-Wilk normality test ($\alpha = 0.05$).

3. Results

3.1. Exposure to model compounds

Upon injection of pure 1,4-NQ solution ($2 \mu\text{g cm}^{-2}$) or 1,4-NQ ($2 \mu\text{g cm}^{-2}$) with copper (Cu(II), $0.2 \mu\text{g cm}^{-2}$), a sharp increase of m/z 159.04, corresponding to protonated 1,4-NQ, was observed, both with the cells present and in the acellular blank (figure 1). The gas phase concentration of 1,4-NQ was not influenced by the addition of Cu(II). The maximum signal intensity was measured in the presence and absence of cells. In the case of the acellular control, the 1,4-NQ signals slowly decreased to a roughly stable concentration of 30 ppbv due to evaporation of 1,4-NQ from DPBS. The evaporation rate corresponded to about $4\% \text{ h}^{-1}$ of the injected amount of 1,4-NQ. The initial peak was most probably due to incomplete mixing right after injection and residual 1,4-NQ on the walls of the Teflon tubes or in the needle. In the presence of cells, the maximum signal intensity reached was 36.4% higher than in their absence, most likely because 1,4-NQ is diluted into a

higher DPBS volume during the control experiments. After this maximum, the 1,4-NQ signal decreased very rapidly and steadily in the presence of cells, even far below the steady-state level reached in the control experiment. This suggests an additional removal mechanism of 1,4-NQ in the apical liquid in presence of BEAS-2B cells. Indeed, we observed by UV–VIS measurements a fast decrease of the concentration of 1,4-NQ in the apical compartment (80% within 60 min, figure 1(B)), while no 1,4-NQ was measured in the basal compartment. This indicates an internalization of 1,4-NQ by the BEAS-2B cells.

Injection of Cu(II) alone into DPBS or onto the cells did not induce any VOC emissions (figure 2). In contrast, injection of 1,4-NQ (figures 2(B) and 2(C)) induced a fast release of compounds detected as $\text{C}_9\text{H}_{11}\text{O}^+$ (m/z 135.08) and $\text{C}_7\text{H}_7\text{O}^+$ (m/z 107.06) ions. In the presence of cells, peak intensities corresponded to 1.218 ± 0.060 ppbv and 0.207 ± 0.024 ppbv, respectively. Similar emissions were observed upon injection of a high dose of H_2O_2 ($100 \mu\text{g cm}^{-2}$), provoking peak intensities of 0.764 ± 0.075 and 0.225 ± 0.053 ppbv of m/z 135.08 and 107.06, respectively. While the co-injection of Cu(II) with 1,4-NQ did not affect these two ions, a strong increase of the $\text{C}_2\text{H}_4\text{N}^+$ signal (m/z 42.03) was observed. This signal was seen neither in the acellular nor in the cellular control experiments. The intensity of this VOC increased 507-fold compared to injection of 1,4-NQ alone, and 225-fold compared to H_2O_2 ($100 \mu\text{g cm}^{-2}$) demonstrating a synergistic effect of 1,4-NQ and Cu(II) (figures 2(a) and 2(c)).

3.2. Wood burning PM and α -pinene SOA

Filter samples from wood-burning experiments (WB-fresh and WB-aged) and α -pinene SOA (fresh and 24 h-aged) were extracted in water. The resulting extracts were injected onto the BEAS-2B cells or only into DPBS (acellular control) in the same way as described for the test compounds. Two concentrations, 0.1 and $0.01 \mu\text{g cm}^{-2}$ were applied for the α -pinene SOA extracts.

Similar to the case of model compounds (1,4-NQ, Cu(II) or H_2O_2), the cell exposure to these PM extracts induced the release of biomarkers detected at m/z 42.03, 107.06 and 135.08. PM extracts and test compounds except for Cu(II) showed similar levels for the m/z 107.06 signal (0.04 to 0.23 ppbv) and the m/z 135.08 signal (0.5 and 1.2 ppbv), as shown in figures 3(E) and (F). The m/z 135.08 peak has the highest signal in all cell treatments ($p < 0.05$) except when only Cu(II) was applied. The increase of the m/z 42.03 signal was higher than for 1,4-NQ and Cu(II) alone, but much lower than for the Cu(II)/1,4-NQ mixture. A higher dose of α -pinene SOA consistently resulted in a higher release of all m/z signals plotted in figure 3.

While fresh WB exhaust solely released the compounds detected at m/z 107.06 and 135.08, exposure

to extracts of aged WB exhaust and α -pinene SOA resulted in the release of additional compounds detected at m/z 41.04, 57.07 and 71.09 (figure 3). In particular, α -pinene SOA extracts induced quite strong emissions of the signals detected at m/z 41.04 (3.9–26.7 ppbv), 57.07 (32.3–130.9 ppbv) and 71.09 (0.6 ppbv). The 24 h aging of α -pinene SOA did not modify the VOC emissions, with the exception of the compounds associated with m/z 41.04 at the dose of $0.1 \mu\text{g cm}^{-2}$, for which a significantly ($p = 0.006$) higher response was observed. It should be noted that the signals at m/z 41.04, 57.07 and 71.09 increased upon injection on both the acellular control and BEAS-2B-cells. In both cases, the time trends were similar to those shown in figures 2(A) and (B), whereby the control experiments showed lower intensity. We explain the appearance of these signals with the decomposition of SOA in the DPBS. The lower signal in the acellular experiment may be explained by a higher dilution in the wells filled with DPBS compared to the small amount of DPBS on the cell surface in the cell experiments.

3.3. Cytotoxicity

Cytotoxicity was assessed by measuring LDH, a cytosolic enzyme released by dead cells. For this analysis, the applied doses of Cu(II) and 1,4-NQ were reduced by a factor of two. Treatment of the cells with 1,4-NQ and Cu(II) induced a significantly ($p < 0.005$) higher LDH release (39.2% and 17.5% for 1,4-NQ and Cu(II), respectively) compared to the control (8.3%, figure 4(A)). Co-treatment with 1,4-NQ and Cu(II) did not significantly increase the release of LDH (40.6%) compared to 1,4-NQ alone. Filter extracts from wood burning (WB-fresh, WB-aged) and fresh α -pinene SOA also significantly ($p < 0.01$) increased cytotoxicity compared to the control. A tenfold increase of the α -pinene SOA dose did not significantly increase cytotoxicity further. Values for H_2O_2 , used as a positive control to induce cytotoxicity (34.4%), were not significantly different from those of 1,4-NQ despite a hundred times higher dose. It should also be noted that the doses of H_2O_2 and 1,4-NQ were 100 and 10 times higher than those of the filter extracts.

Figure 4(B) shows the relationship between measured emissions of VOCs and cytotoxicity. Except for the experiment with Cu only, an increase in the m/z 107.06 signal with increased LDH release was observed for all tested compounds and extracts, however, with a large signal variability at low cytotoxicity. For the signals observed at m/z 42.03 and m/z 135.8, no correlation with LDH release was detected (not shown).

4. Discussion

Upon injection of 1,4-NQ on the BEAS-2B cells, the concentrations of 1,4-NQ in the gas phase and

apical liquid decreased by about 80% within one hour indicating uptake of this compound by the cells. Cells under stress induced by model compounds (H_2O_2 , Cu(II), 1,4-NQ or their combination), as well

as by fresh and aged wood combustion emissions and by α -pinene SOA showed a prominent peak of VOCs detected at m/z 42.03, 107.06 and 135.08 within minutes after injection, followed by a rapid decrease

of the signal by 70%–80% within about 30 min. This distinct pattern could indicate that these compounds originated from reactions at the cell surface (e.g. peroxidation of the membrane [37]). We did not observe any delayed strong signals after cellular uptake of the compounds, which would indicate intracellular reaction products. High signals at m/z 41.04, 57.07 and 71.09 appeared in the α -pinene SOA experiments, however, in both the BEAS-2B cells and the control experiments. Therefore, these signals probably originated from compounds formed by the SOA decomposition in DPBS and not as a response from the cells.

The high mass resolution of the PTR-ToF-MS instrument allows obtaining the chemical formula of the measured m/z signals. A potential assignment of the m/z signal to a compound, together with supporting literature, is provided in table 2. The signal at m/z 42.03 is tentatively assigned to acetonitrile. Even though this compound has been measured in the breath of non-smokers (5–10 ppbv) [38], its endogenous origin is questioned, as it might originate from passive smoking [39]. In our study, its concentration in the cell experiments was always higher than in the controls. Furthermore, peak concentrations in control experiments were below 100 pptv, which we interpret as the maximum level of contamination from laboratory air or other sources of sample contamination with acetonitrile. Based on these findings, we conclude that acetonitrile was released from the stressed cells. We attribute the m/z 107.05 and m/z 135.08 signals to benzaldehyde and dimethylbenzaldehyde, respectively, although this still needs to be

confirmed with a more specific chemical-analytical method (e.g. GC/MS). Aldehydes are generated as a secondary product of lipid peroxidation [40]. Benzaldehyde has been assigned in some breath analysis studies to exogenous factors (e.g. smoking) [41, 42], while others claimed an endogenous origin due to cancer [43, 44]. Dimethylbenzaldehyde was observed in the headspace of breast cancer cell lines [45]. While the cellular emissions of dimethylbenzaldehyde were roughly 5 times higher than those of benzaldehyde, the emissions of both compounds did not substantially depend on the aerosol dose or type. A tenfold higher dose of α -pinene SOA increased the VOC emissions only by a factor of two to three. The high dose of $100 \mu\text{g cm}^{-2}$ H_2O_2 did not trigger significantly higher VOC emissions compared to 1000 to 10 000 times lower doses of wood combustion aerosol and α -pinene SOA. This is an indication of a high reactivity of OAs with cell membranes. We note that doses of 0.01 and $0.1 \mu\text{g cm}^{-2}$ of aerosols to the cells are relevant as they correspond approximately to 0.3 to 3 d of inhalation at an ambient PM concentration of $100 \mu\text{g m}^{-3}$. Exposure to extracted aerosols from wood combustion induced a similar cytotoxicity as reported by Krapf *et al* [46] for directly deposited wood combustion aerosols on BEAS-2B cells at equivalent doses. The content of particle-bound reactive oxygen species (PB-ROS given as H_2O_2 equivalents) in these particles reported by Krapf *et al* [46] and Zhou *et al* [29] yields an ROS dose of 0.1 – 1 ng cm^{-2} and 2 – 8 ng cm^{-2} for WB-fresh and WB-aged at a total dose of $0.1 \mu\text{g cm}^{-2}$ extract, respectively. These small

doses are sufficient to induce measurable emissions of biomarkers and significant cytotoxicity in BEAS-2B cells.

In summary, we have shown that ambient particles react with epithelial cells and induce biomarker emissions that could be measured *in-situ* by PTR-MS. We demonstrate the potential of our method to elucidate the cellular mechanisms by which aerosols affect the respiratory tract. We have shown that the cellular response to exposure is rapid, which highlights the necessity of using an online and sensitive detection method. The injection of different compounds or filter extracts led to the emission of specific biomarkers, which may be linked to different

sources of toxicity. For example, acetonitrile was not emitted by 1,4-NQ, but by a 1,4-NQ/Cu mixture. As our method is not destructive, it also allows for subsequent cellular assays (cytotoxicity, inflammation, gene expression, etc.). Recent research has found that BEAS-2B cells respond differently to particle exposure compared to fully-differentiated human bronchial epithelia (HBE) [46, 51]. Therefore, we also recommend further experiments with ALI cultures of HBE producing mucus and antioxidants, as they will provide additional information on the cellular reactivity, as well as the metabolic and chemical pathways leading to cellular release of VOCs. Moreover, HBE derived from donors with pre-existing pulmonary

Table 2. List of the m/z of interest, chemical formula of ions, tentative identification and references from literature reporting m/z observed in humans or cells.

Measured m/z	Chemical formula of ion	Tentative identification	Reference
41.039	$C_3H_5^+$	Unspecific fragment ion	
42.034	$C_2H_4N^+$	Acetonitrile	[47]
57.071	$C_4H_9^+$	Unspecific fragment ion	[48]
71.087	$C_5H_{11}^+$	Unspecific fragment ion	[49, 50]
107.051	$C_7H_7O^+$	Benzaldehyde	[41, 43, 44]
135.084	$C_9H_{11}O^+$	Dimethylbenzaldehyde	[45]

disease will allow deciphering normal and diseased responses [52]. This may lead to the creation of a library of cellular VOC biomarkers and their corresponding sources of PM.

Experimental studies in humans to evaluate adverse effects from exposure to air pollution are difficult to perform, foremost for ethical reasons. Non- or minimally invasive methods, also applicable to vulnerable populations, like persons with pre-existing disease, pregnant women, newborns and young adults are highly desirable. Today, such investigations include measurements in urine and blood samples [53–55]. Thereby, oxidative stress is assessed mainly via analysis of inflammatory products in blood samples, or by measuring their metabolites or markers of DNA damage or lipid oxidation excreted into urine. Direct evaluation of lung inflammation is also possible by means of sputum induction allowing quantification of inflammatory cells and mediators [56]. In recent years, exhaled breath analysis has been increasingly used as a new frontier in lung diagnosis and health inspections, and to assess metabolic or (patho-)physiological processes [57, 58]. As a quick and non-invasive method, exhaled breath analysis has an enormous potential to monitor inflammation as well as oxidative stress in lungs. In future studies, the comparison of breath analysis investigations of specific compounds with more established methods (e.g. urine, sputum and blood analysis) will lead to scientific progress in the field. Therefore, the methodology described in this paper can contribute to detect the effect of exposure to ambient PM in breath owing to its potential in developing rapid, noninvasive, and inexpensive screening tools.

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