Evaluation of peroxidative stress of cancer cells *in vitro* by real time quantification of volatile aldehydes in culture headspace

Violetta Shestivska[1], Abigail V. Rutter[2], Josep Sulé-Suso[2], David Smith[2], Patrik. Španěl[1*].

[1] J. Heyrovsky Institute of Physical Chemistry of Science, Academy of Science of the Czech Republic, Dolejškova 3, 18223 Prague 8, Czech Republic.
[2] Institute for Science and Technology in Medicine, School of Medicine, Keele University, Thornburrow Drive, Hartshill, Stoke-on-Trent ST4 7QB, UK

Abstract

RATIONALE: Peroxidation of lipids in cellular membranes results in the release of volatile organic compounds (VOCs), including saturated aldehydes. The real-time quantification of trace VOCs produced by cancer cells during peroxidative stress presents a new challenge to non-invasive clinical diagnostics, which we have met with some success, as described in this paper.

METHODS: A combination of selected ion flow tube mass spectrometry (SIFT-MS), a technique that allows rapid, reliable quantification of VOCs in humid air and liquid headspace, and electrochemistry to generate reactive oxygen species (ROS) *in vitro* has been used. Thus, VOCs present in the headspace of CALU-1 cancer cell line cultures exposed to ROS have been monitored and quantified in real time using SIFT-MS.

RESULTS: The CALU-1 lung cancer cells were cultured in 3D collagen to mimic *in vivo* tissue. Real time SIFT-MS analyses focused on the volatile aldehydes, propanal, butanal, pentanal, hexanal, heptanal and malondialdehyde (propanedial), that are expected to be products of cellular membrane peroxidation. All six aldehydes were identified in the culture headspace, each reaching peak concentrations during the time of exposure to ROS and eventually reducing as the reactants were depleted in the culture. Pentanal and hexanal were the most abundant, reaching concentrations of a few hundred parts-per-billion by volume, ppbv, in the culture headspace.

CONCLUSIONS: The results of these experiments demonstrate that peroxidation of cancer cells *in vitro* can be monitored and evaluated by direct real time analysis of the volatile aldehydes produced. The combination of adopted methodology potentially has value for the study of other types of VOCs that may be produced by cellular damage.

* *Correspondence to*: Patrik Španěl, J. Heyrovsky Institute of Physical Chemistry, Academy of Science of the Czech Republic, Dolejškova 3, 18223 Prague 8, Czech Republic.
E-mail: patrik.spanel@jh-inst.cas.cz

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Lipid peroxidation of cellular membranes results in the release of different volatile organic compounds (VOCs) such as aldehydes, alkenes and hydrocarbons.[1, 2] Their appearance in plasma, exhaled breath, saliva and sweat may act as biomarkers of diseases that involve increased oxidative stress of cells.[3-6] A comprehensive metabolomic investigation of trace VOCs produced when cells are exposed to peroxidative stress represents a challenge to non-invasive clinical diagnostics. [7, 8] Such is the focus of the present study.

The chosen approach was to perform real-time quantification of volatile aldehydes produced by the action of reactive oxygen species (ROS) on cancer cell lines *in vitro*. The ROS are produced electrochemically and the VOCs analysis is by selected ion flow tube mass spectrometry, SIFT-MS, a combination of techniques that could be used for real-time observations of VOCs released by other organisms exposed to ROS such as bacteria, mammalian cells and model membranes. The immortal lung cancer cell line CALU-1 has been used to test and develop this combination of techniques. We have given considerable attention to these cancer cell lines, specifically their emission of acetaldehyde in cultures, [9] as a prelude to the study of this aldehyde and other VOCs in exhaled breath as a potential pre-diagnostic step for lung cancer diagnosis and also as a method for non-invasive monitoring of cancer patients during chemotherapy and/or radiotherapy and during follow-up. However, it should be stressed that the present study was not focused on the search for VOCs in exhaled breath but rather a contribution to the study of fast running peroxidation processes in living organisms by ROS and real time VOCs analysis by SIFT-MS. In this study we produced ROS in the presence of the CALU-1 cells cultured in the presence of collagen to study peroxidative stress by real time SIFT-MS quantification of volatile aldehydes that appeared in the culture headspace. To the best of our knowledge, this combination has not been investigated previously.

Aldehydes - peroxidation stress biomarkers

Most bioactive saturated aldehydes produced during lipid peroxidation of cell membrane are volatile at physiological temperature and are released into the headspace,[6] but most are unstable in both the liquid and gaseous phases. It has been shown that these aldehydes are the products of the peroxidation of omega-3 and omega-6 polyunsaturated fatty acids contained in cell membranes. Omega-3 fatty acids are formed in mammalian cells from the ‘essential’ 18 carbon alpha-linolenic acid (ALA;C18:3 n-3) and include eicosapentaenoic (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3), while omega-6 acids are metabolites of linoleic acid (LA; C18:2 n-6) and include arachidonic acid (AA; C20:4 n-6) which plays a key role in cell signaling.[10] Pentanal and hexanal have been reported as the main peroxidation products of ω-6 polyunsaturated fatty acids (PUFA), and propanal was observed mainly from ω-3 PUFA peroxidation. [11] The production of hexanal is connected with linoleic and arachidonic acid peroxidation.[12] C_3-C_7 aldehydes and malondialdehyde (propanedial) have been quantified by SIFT-MS in the headspace of cancer cell cultures exposed to peroxidative stress.[6, 13]

Background of Selected ion flow tube mass spectrometry

SIFT-MS is an analytical technique for real time, on-line analysis of the small volatile molecules in ambient air. It has been developed by Smith and Španěl for the quantification of volatile compounds in ambient air and human breath with the primary objective of utilizing this technique in medicine and environmental research. [13, 14] The basic principle of SIFT-MS for real-time analysis and quantification of trace gases in air samples has been described in detail previously.[14, 15] SIFT-MS exploits selected precursor positive ions, H_3O^+, NO^+ or $O_2^{+\bullet}$, to ionize the trace gas molecules in a sample that is introduced directly into an inert carrier gas (usually helium) in a flow tube. Absolute quantification is achieved by knowing the rate coefficients and ion products of the selected precursor ion with the analyte trace gas molecules in the air sample. The full scan (FS) mode of SIFT-MS operation is first used to identify the trace compounds in the sample by their characteristic product ions from the analytical ion-molecule reactions. The multi-ion monitoring (MIM) mode is then used for accurate quantification by rapidly switching the downstream analytical mass spectrometer between the *m/z* values of the precursor ion (*m/z* 30) and the product ions appropriate for each analyte based on a SIFT-MS kinetics library. The concentrations of each compound are then calculated from the recorded ion count rates, reaction rate coefficients, flow tube pressure and carrier gas and sample flow rates. [15] In this way, several trace compounds can be accurately and simultaneously quantified in an air mixture, including very humid samples such as exhaled breath and the headspace of aqueous liquids, down to about 1 parts-per-billion by volume, ppbv, in one second of integration time of the product ion counts. Thus, SIFT-MS has been applied to study the VOCs in human breath, those VOCs emitted by bacterial and cell cultures and foods, and the products of explosives.[16-21] The ease of real-time data acquisition and analysis of SIFT-MS makes it eminently suitable for the present study, especially because sample collection and pre-concentration of the unstable trace gases in the humid headspace above the cell culture headspace are avoided.

Electrochemically induced peroxidation stress in cancer cells

The most frequent methods of peroxidation induction are by either autoxidation processes or by hydrogen peroxide and Fenton reagents. [22, 23] Electrochemical peroxidation methods are valuable for the dynamic production of radicals and ROS. For example, direct electron transfer peroxidation coupled with electrospray mass spectrometry has been shown to be a promising technique for the study of peroxidation processes and the stability of organic molecules in liquid samples [24, 25], and indirect peroxidation of molecules by ROS and hydrogen peroxide. [26] Electrochemical reduction of molecular oxygen has been shown to lead to the oxidation of various organic substrates [27-29] and indirect electrochemical oxidation has been used recently in combination with liquid chromatography mass spectrometry.[30] Note that free radicals can be studied in real time using ESR [31]or electrochemical sensors [32]. However, the objective of the present work was to identify volatile metabolites caused by the interaction of free radicals with living cells.

For electrochemical studies such as the present work, a potentiostat in an electrochemical cell maintains the potential of the working electrode (W) at a pre-set value with respect to a fixed reference electrode (R). The instrument does so by passing the necessary current between the working electrode and the third counter electrode (C). The current driven between R and W depends upon the potential difference between them. [28] The potentials needed for

electrochemical ROS production depend on the electrode materials. It has been reported that for a gold electrode the reduction of molecular oxygen to the superoxide anion requires a potential of -0.75 V, and reduction to the singlet peroxide anion requires -2.05 V. [30] A proposed scheme for the generation of ROS using electrochemistry is shown by reactions 1-5.

Two-electronic reduction of oxygen to hydrogen peroxide, H_2O_2, is effected at the cathode (reaction 1). At the anode, water oxidation proceeds to form the $OH^{\bullet}$ radicals as intermediates (reaction 2). [28]

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \quad (1)$$
$$2H_2O \rightarrow 2OH^{\bullet} + 2H^+ + 2e^- \quad (2)$$

$HO_2^{\bullet}$ radicals, which can contribute to the oxidation of organic compounds [28], are also produced at the anode:

$$H_2O_2 \rightarrow HO_2^{\bullet} + H^+ + e^- \quad (3)$$
$$H_2O_2 + OH^{\bullet} \rightarrow HO_2^{\bullet} + H_2O \quad (4)$$

Experimental

Electrochemical measurement

A homemade potentiostat controlled by custom software to establish and control the desired potentials within the electrochemical cell was used. For the purpose of the experiments, the potentiostat was equipped with three electrodes: the platinum working electrode, the platinum plate counter electrode M241Pt and the glass reference electrode filled with Ag/AgCl 3 M (all electrodes supplied by *R*adiometer Analytical, Lyon, France); see Fig. 1B. The supporting electrolyte was 0.1mM phosphate buffer (Sigma Aldrich, Taufkirchen, Germany) prepared in 40 mL of deionized ultra-pure water (Sigma Aldrich) at a pH of 7.4, which was bubbled with dry air (zero grade compressed air from a 200 bar cylinder; BOC Gas, Manchester, UK) during the experiments. Electrochemical indirect peroxidation was performed at potentials ranging from -0.75 to -2.4 V whilst the current was continuously registered in the range 0.012 to 7.6 mA. All experiments were performed at room temperature.

Electrochemical generation of radicals

To achieve electrochemical indirect peroxidation of the CALU-1 cancer cell in cultures and real-time quantification by SIFT-MS of the VOC products of peroxidation in the culture headspace, a hermetically sealable glass electrochemical cell with 5 inlets for three electrodes, dry air injection and direct gas phase sampling was constructed. Non-small cell lung cancer cells CALU-1 were cultured in a 3 D collagen model, as previously described, [9] directly in the glass electrochemical cell. This allows separation of the cancer cells from the electrodes and physiological cell growth under conditions closer to human tissue.

The seeded number of cells was close to 10^6 as determined by a standard trypan blue exclusion assay. The total final volume of the culture was 10 mL. The dry air was introduced to compensate for the loss of headspace by the analytical sampling procedure and thus to maintain the pressure in the sealed vessel close to atmospheric. The electrochemical cell was hermetically sealed and the potentiostat was then switched on to generate the free radicals

that induce peroxidation stress in the CALU-1 cells, producing volatiles in the headspace (60 mL) that are quantified by SIFT-MS in real time. The final cell count after the experiments were carried out was close to 2×10^6 and the reproducibility of three independent experiments was ($\pm$ 0.04×10^6). This was determined through the digestion of the 3D collagen model using collagenase from Clostridium histolyticum (working concentration 1000 units/ mL), which released the cells and allowed them to be counted using the standard trypan blue exclusion assay.

Cell culture protocol

CALU-1 is a lung squamous carcinoma cell line (European Collection of Cell Cultures, Salisbury, UK). These cells were cultured as a monolayer in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% sodium pyruvate, 1% non-essential amino acids, 1% penicillin-streptomycin according to the provider's instructions (Sigma-Aldrich, Gillingham, UK). Monolayer cultures were maintained in T75 culture flasks in 5% CO_2 and 37 °C humidified incubator. The cells were routinely passaged before reaching confluence using trypsin-EDTA (ethylenediaminetetraacetic acid) solution, incubating for 5 minutes at 37 °C. The cell culture suspension was spun for 5 minutes at 1200 rpm. The supernatant was then discarded, the pellet then being dislodged and re-suspended in fresh DMEM. CALU-1 viability was assessed using the trypan blue exclusion method, to ascertain cell count and survival.

3D collagen models were implemented to mimic *in vivo* tissue more accurately. The models were constructed from rat tail collagen type I (10.59 mg/mL, BD Life Science, Oxford, UK), forming a hydrogel with a final concentration of 1.5 mg/mL, based on previously described work. [33] The 3D model is constructed from 10 mL neutralized acetic acid extracted collagen, 10X phosphate buffered saline (PBS) and the cell pellet re-suspended in liquid form. This 10 mL suspension is then transferred into the glass electrochemical cell and the CALU-1 cells were then seeded at 10^6 and incubated at 37°C for 2 hours. Following this incubation time, the collagen polymerized to form a fine lattice of fibrils in a hydrogel 3D model sitting in the base of the electrochemical cell. The hydrogel was then topped up with 10 mL DMEM medium and incubated for 16 hours. Before the electrochemical indirect peroxidation was applied to the culture, 5 mL of medium was discarded and 40 mL of fresh PBS were added. After cell peroxidation, CALU-1 viability was assessed again using the trypan blue exclusion method. The cells were then cultured again in monolayer in DMEM in plastic flasks to test their ability to replicate. The final cell count after the experiments were carried out was 2.12×10^6.

SIFT-MS quantification of volatile metabolites using NO^+ reagent ions

A *Profile 3* SIFT-MS instrument (Trans Spectra Limited, Newcastle under Lyme, UK) was used for real time quantification of the VOCs in the headspace of three scenarios: (i) the collagen gel with phosphate buffer 0.1 M, pH 7.4 electrolyte, (ii) the CALU-1 cells with the gel and electrolyte, and (iii) the CALU-1 cells with the gel and electrolyte when exposed to the ROS, as is clearly explained later. Headspace samples entered the SIFT-MS carrier gas/reactor via a heated calibrated capillary (0.18 mm), at a flow rate of 10 mL/min. FS analytical mass spectra were obtained using the three reagent ions (NO^+, H_3O^+ and $O_2^{+\bullet}$) for each of the three scenarios (i), (ii) and (iii) over the *m/z* mass spectral range of 10–150, which

was wide enough to encompass all the reagent and product/analyte ions. This is common practice in SIFT-MS measurements and is carried out to choose the most appropriate reagent ion to analyze a given medium. For each reagent ion, five sequential FS spectra were obtained with a total integration time of 60s, which resulted in combination spectra with well-defined spectral peaks.

The analytical spectra obtained using all three reagent ions for the analysis of the situation described by scenario (iii) viz. the gel/cells when exposed to ROS, showed characteristic analyte ions of aldehydes, as can be seen in Fig. 2. What is also clear is the relative complexity of the H_3O^+ and $O_2^{+\bullet}$ reagent ion spectra compared with that for the NO^+ reagent ion. This complexity is due to the production of hydrated reagent ions, and consequently hydrated analyte ions, when using H_3O^+, and to serious fragmentation of polyatomic analyte molecules when using $O_2^{+\bullet}$. This results in some overlaps of analyte ions of different character that have the same nominal *m/z* values, which can make quantification of trace compounds both difficult and inaccurate. Note the simplicity of the NO^+ reagent ion spectrum in which each neutral aldehyde is identified by a single analyte ion, these being recognized as propanal, butanal, pentanal, hexanal, heptanal and malondialdehyde. So, consistent with the precedence set by previous work on the analysis of aldehydes in complex media,[34, 35] we chose to utilize NO^+ as the reagent ion in the present studies**.** It is, however, pertinent to note that inspection of the corresponding H_3O^+ and O_2^+ spectra and a knowledge of ion chemistry accumulated over many years gave conclusive supporting evidence that these compounds are indeed aldehydes and not, for example, isobaric ketones. Two different experiments were carried out, one in which the VOCs were allowed to accumulate in the headspace following a short exposure time to ROS and the headspace analysed periodically post exposure, and the other in which the cells were exposed for a longer period to ROS while the headspace was continuously analysed, continuing after the production of ROS was stopped. In both experiments the pressure in the cell was maintained at atmospheric by the introduction of dry air (see Fig. 1C).

The ion molecule reaction kinetics data required for the quantification of these aldehydes using NO^+ reagent ions are given in previous publications [35, 36] and are summarized in Table 1. These data have been utilized in several previous *in vitro* studies in food science, breath research and cell reactions studies. [9, 37-39] Direct SIFT-MS quantification of malondialdehyde generated by peroxidation of cancer cells has been recently reported.[13] The concentrations of the several aldehydes were measured using the MIM mode. The list of the quantified aldehydes, the rate coefficients for their reactions with NO^+ ions and the *m/z* values of the product/analyte ions that are entered into the SIFT-MS kinetics library used to calculate absolute concentrations are given in Table 1. It should also be noted that the $NO^+(H_2O)$ and $NO^+(H_2O)_2$ hydrated ions are routinely included as reagent ions for accurate analyses, but these hydrates are only a small percentage (<5%) of the total reagent ions, as can be seen in Fig. 2B.

RESULTS AND DISCUSSION

SIFT-MS quantification of aldehydes in the headspace of cancer cells

At the onset of these experiments it was recognized that the cell growth medium represents a relevant part in any cell culture experiment because, potentially, it can release VOCs into the culture headspace and confuse cellular production measurements. This could also be the case for the collagen used in the present experiments. Thus, headspace measurements were carried out of the VOCs in three separate scenarios, given as (i), (ii) and (iii) previously.

Control collagen without cells (scenario (i)) was cultured for 16 h under the same conditions as is normal for cell cultures. Following this incubation period, 40 mL of electrolyte (phosphate buffer 0.1M pH 7.4) was added as the supporting electrolyte. When the headspace was analysed using NO^+ reagent ions, the analytical spectrum was as expected for an aqueous medium except for the appearance of an obvious peak at *m/z* 99. This peak persisted after the gel was exposed to electrochemical peroxidation by ROS by applying -2.4 V for 5 minutes to the working electrode, but no other new peaks were seen except for small signals indicative of acetic acid and small peaks due to unidentified trace compounds. This peak at *m/z* 99 can be attributed to the presence of hexanal, as indicated in Fig. 2B. In Fig. 2A when H_3O^+ reagent ions are used, a peak appears at *m/z* 101, which is a sure sign that there is a compound present in the headspace of molecular weight 100. However, it must be recognized that the compound signaled in the gel headspace alone may be another compound that is also of molecular weight 100. This is significant because, as we see later, hexanal is apparently produced in the peroxidation of the CALU-1 cancer cells and therefore, strictly speaking, it should be accounted for in interpreting the cellular experiments.

Sample mass spectra are shown in Fig. 3 for the SIFT-MS analysis using injected NO^+ reagent ions of the headspace of CALU-1 cancer cells in the gel/electrolyte combination (A) before exposure to the ROS and (B) after exposure. The spectrum in (A) shows the $NO^+(H_2O)_{0,1,2}$ reagent ions and smaller signals of hydrated hydronium ions, always present at low levels when using NO^+ reagent ions, together with small impurities of acetone (*m/z* 88), ethanol (*m/z* 45 and 47 and their hydrates) and the supposed hexanal at *m/z* 99. Small levels of acetone and ethanol seem inevitably to be present in these and previous experiments on cell cultures [40]. Their origins are undetermined, but such trace levels are inconsequential to the present experiments. The spectrum in (B), following exposure to ROS, also shows the characteristic analyte ions of acetaldehyde (*m/z* 43), propanal (*m/z* 57), butanal (*m/z* 71), pentanal (*m/z* 85), hexanal (*m/z* 99) and heptanal (*m/z* 113) [6] and small signals of *m/z* 89 and 102 attributed to malondialdehyde, and *m/z* 90 and 108 attributed to acetic acid. [13] It is clear that NO^+ analyte ions can be used to identify and monitor the appearance of these aldehydes in real time in the culture headspace during exposure of the cells to peroxidative stress. Such data are now presented below.

Quantification of aldehydes released by CALU-1 cells subjected to peroxidation stress

The production of aldehydes when cells in culture are subjected to peroxidation stress conforms to the results from previous SIFT-MS studies.[6, 13] In the first experiments, the concentrations of individual aldehydes in the CALU-1 culture headspace was followed as the potentiostat was switched on for a limited period only and the headspace concentrations of

the aldehydes measured at later times. First, however, the headspace above the gel/electrolyte headspace was analyzed when exposed to electrolytic excitation and ROS production by applying a potential of -1.75 V to the working electrode for 5 minutes (current 0.54 mA) after which the voltage was removed and the headspace was immediately sampled and analyzed for the six aldehydes. Sampling and analysis were then carried out after a further 10 minutes and then again after a further 60 minutes. Analyses were performed using the MIM mode of SIFT-MS and each sampling period was for 60 seconds. The gel/electrolyte/cells culture was analyzed before excitation and ROS production and then after excitation and ROS production using the above timing protocol. To repeat, throughout each analysis, dry air was bubbled through the cultures to maintain laboratory air pressure in the cells. This procedure inevitably results in some dilution of the headspace aldehydes, but this is not significant at the sample flow rate of only 10 mL/min. Figure 4 shows the mean concentrations of each aldehyde in the headspace over the 60 s analysis time in parts-per-billion by volume, ppbv, as vertical bar charts.

Inspection of these bar charts reveals that the headspace concentrations of all the aldehydes fall over time, presumably because of either metabolism by the cancer cells due to aldehyde dehydrogenases expression [41] or chemical reactions possibly forming diols (hydrates). Pentanal and heptanal are marginally the major products of peroxidation stress in this scenario, reaching about 200 ppbv in the headspace. Increasing levels of these aldehydes suggest the presence of ω-6 fatty acids in the membranes of the CALU-1 cells.[11] The production of propanal suggests the presence of ω-3 polyunsaturated fatty acids in the cellular membranes [11] Malondialdehyde is usually considered as the most common biomarker of peroxidation stress in liquid phases [42, 43] but in these peroxidation scenario its concentration was relatively low, reaching about 80ppbv. Given the possible uncertainty of hexanal identification, as discussed above, it is seemingly present in both the gel/electrolyte headspace and the gel/electrolyte/cell headspace when they are exposed to the ROS peroxidation. It can be hypothesized that hexanal is being produced via the peroxidation of linoleic and arachidonic acids present in the cell membranes. [12] Actually this aldehyde is at lower concentration when the cells are present, so we hypothesize that it may be toxic to the cells, which may be metabolizing it, thus removing it from the medium during cell growth. The evaluation/comparison of the relative aldehydes production on the basis of their headspace concentrations only can lead to inaccurate conclusions. It should be noted that the headspace concentrations of VOCs are determined by their Henry's Law partition coefficients, which can differ considerably. In other words, a similar headspace concentration can correspond to quite different concentrations in the liquid phase.

As indicated earlier, in the second experimental approach the headspace aldehyde concentrations were monitored continuously during exposure of the cells/media to ROS peroxidative stress and after the ROS production was stopped. In these experiments, the potential of the electrode was first fixed at -0.75 V and then incrementally increased every 5 minutes to a maximum of -2.4 V during which the current increased from 0.012 mA to 7.500 mA. After 30 minutes of excitation the potentiostat was switched off and the headspace analysis continued for a further 15 minutes. Figure 5 shows real-time continuous SIFT-MS quantification of the six aldehydes in the gel/electrolyte/CALU-1 cells culture. As before, the electrolyte was bubbled with dry air during the sampling. These data indicate that aldehyde production in the headspace of cancer cell culture was markedly enhanced by increasing both

the voltage (-2.4 V) and the current (7.5 mA) in the electrolyte, which lead to ROS production. Aldehyde production has a rapid onset when the electrode potential increases beyond about -2V, reaching a maximum of about 600 ppbv for heptanal and about 400 ppbv for pentanal. The headspace concentrations of all the aldehydes peaks then decrease as ROS production, and hence cellular production, slow down whilst losses continue from the headspace due to sampling into the SIFT-MS instrument and metabolism by the cell culture [41]. A control experiment in which the headspace of the gel/electrolyte/CALU-1 cells culture was analysed for 45 minutes without electrochemical excitation resulted in maximal concentrations of hexanal of 40 ppbv and propanal 30 ppbv, with all other aldehydes below 20 ppbv. Another control experiment when electrochemical excitation was applied to collagen gel and electrolyte without cells resulted in a maximum headspace concentration of hexanal of 150 ppbv, with all other aldehydes below 20 ppbv. Note that the absolute aldehyde concentrations are lower in the data obtained by continuous sampling at a potential of -1.7 V (see Fig. 5) than those in Fig. 4 where the headspace was kept static at the same potential for 5 minutes without headspace sampling.

Conclusions

Electrochemical production of ROS in *in vitro* cultures of cells and the real time detection and quantification of volatile aldehydes by SIFT-MS is a powerful combination by which to study cellular membrane peroxidation, as the results of this initial study indicate. For the present study, we chose to investigate the action of ROS on CALU-1 lung cancer cells cultured in 3D collagen to mimic *in vivo* tissue. Direct analysis of the culture headspace revealed the presence of several volatile C_3-C_7 saturated aldehydes, notably pentanal and heptanal, the increasing concentrations of which can be followed in real time as the peroxidation process develops. Malondialdehyde is also produced but at relatively low concentrations. Some suggestions are made as to the likely origins of the various saturated aldehydes and the cellular membrane composition. Clearly, this combination of ROS production and direct, real time analysis of volatile compounds can be applied to the study of peroxidation processes involving normal mammalian cells and other cancer cells cultured *in vitro*. Such would be a valuable contribution to cell biology. More speculative, but nonetheless an exciting possibility, is that some of these aldehydes could be present in exhaled breath of patients suffering from inflammatory disease and might be detected if they are present at measurable concentrations. Only further focused research will show if this could be realized and if breath analysis of these aldehydes can provide valuable support to clinical diagnosis and the efficacy of therapy.

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Table 1. SIFT-MS kinetics library entries for the quantification of aldehydes in humid headspace using NO^+ reagent ions (*m/z* 30).

Name, (molar weight) CAS No	Product ions (*m/z*)	Reaction rate coefficient [10^{-9} cm^3 s^{-1}]	Reference
Propanal (58) 123-38-6	57[a]	2.5	[35]
Butanal (72) 123-72-8	71	3.5	[35]
Pentanal (86) 110-62-3	85	3.2	[35]
Hexanal (100) 66-25-1	99	2.5	[35]
Heptanal (114) 111-71-7	113	2.5	[6]
Malondialdehyde (propanedial) (72) 542-78-9	89,102[b]	3.5	[13]

[a] Corrected for isotopic contribution of ^{18}O isotopologue of $H_3O^+(H_2O)_2$ by subtracting the count rate at *m/z* 55 multiplied by 0.006.

[b] Product ions chosen to minimize overlap according to results from [13].

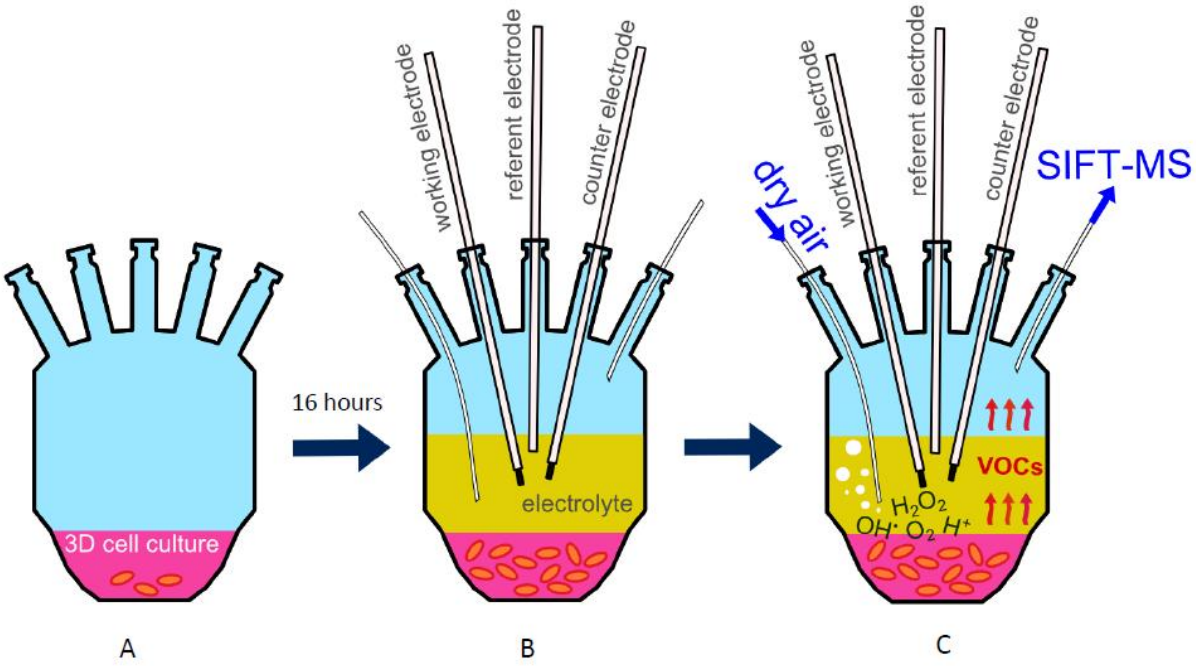

Figure 1 Schematic representation of the experimental apparatus. **A**) CALU-1 cells cultured in a collagen 3D model for a period of 16 hours. **B**) 40 mL of 0.1 M phosphate buffer electrolyte at a pH of 7.4 added above the cell culture. **C**) Shows the final setup of the apparatus in the running mode with the three electrodes, viz. the Pt working electrode, the counter electrode and the Ag/Cl - reference electrode, and the dry air inlet and the inlet for sampling of the headspace into the SIFT-MS instrument.

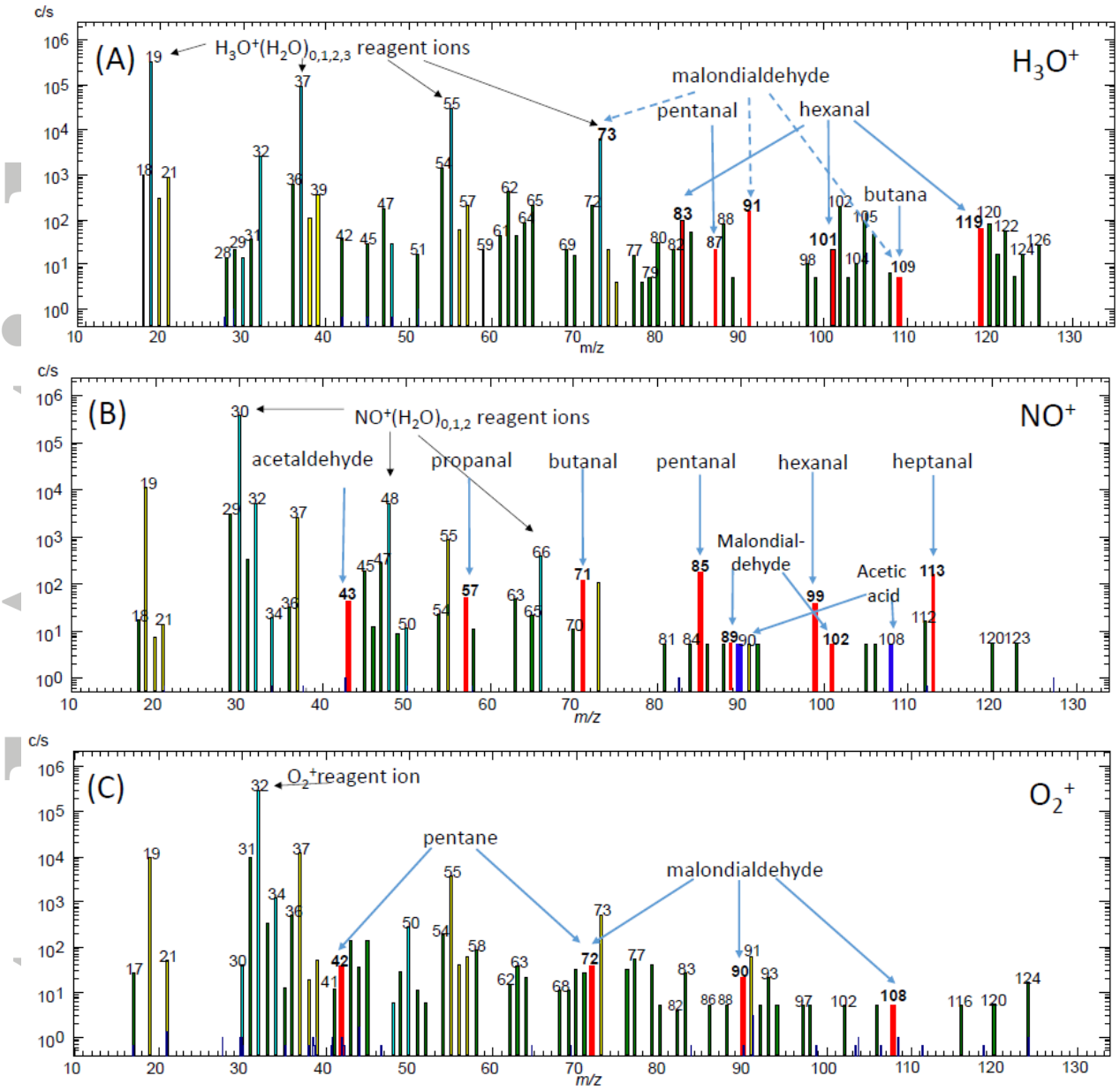

Figure 2. SIFT-MS analytical spectra obtained for the analysis of the headspace of incubated collagen/electrolyte medium with CALU-1 cells exposed to ROS using the reagent ions (A) H_3O^+, (B) NO^+ and (C) $O_2^{+\bullet}$ with -2.4 V applied to the working electrode. All spectra are obtained after CALU-1 cell incubation.

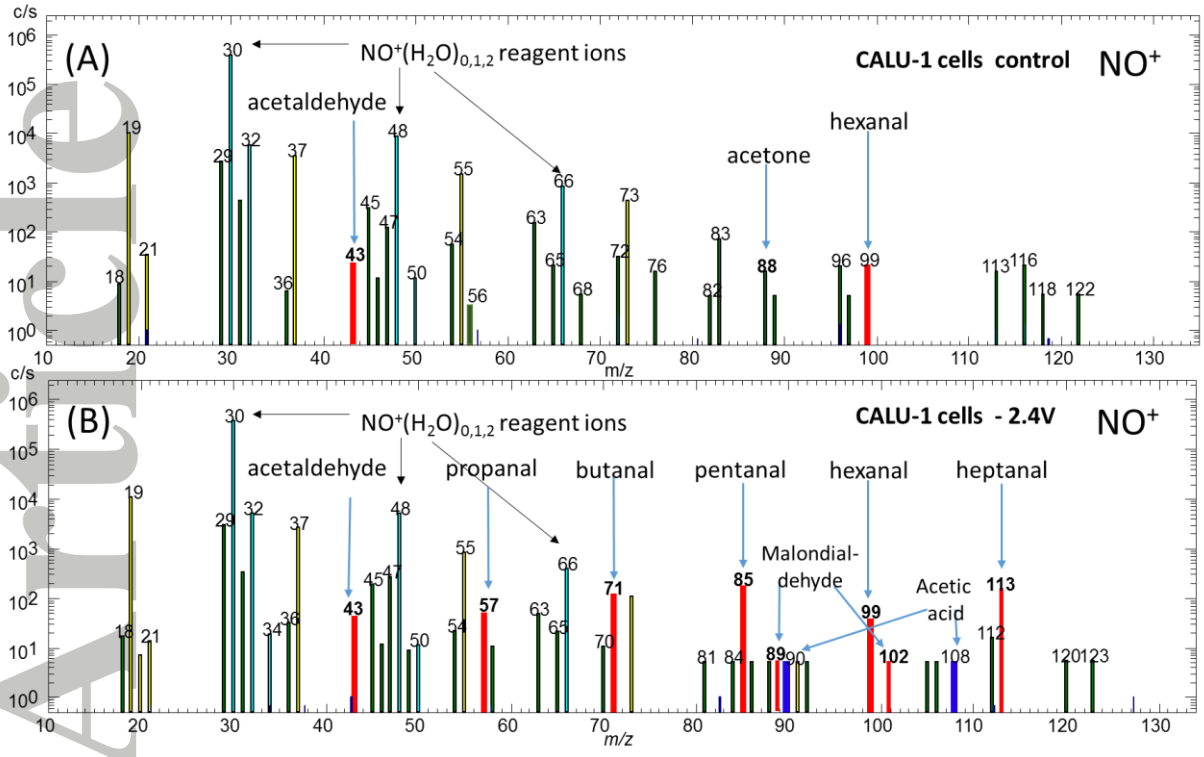

Figure 3. SIFT-MS analytical spectra obtained for the analysis of the headspace of incubated collagen/electrolyte medium with CALU-1 cells using NO^+ reagent ions, (A) before and (B) after exposure to electrochemical peroxidation by ROS. All spectra are obtained after CALU-1 cell incubation.

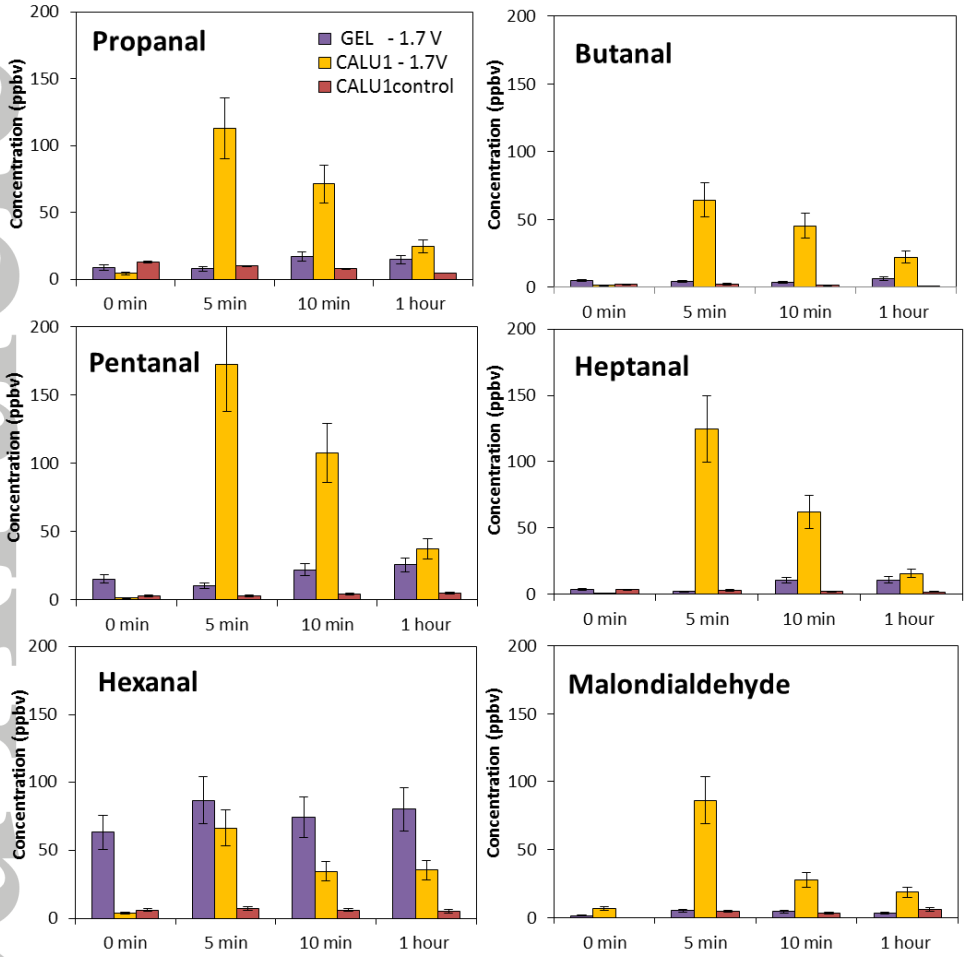

Figure 4. Aldehyde concentrations in headspace of gel/electrolyte/medium after exposure to ROS (GEL -1.7) and the gel/electrolyte/CALU-1 cells before exposure to ROS (CALU-1control) and after exposure to ROS (CALU-1 -1.7) according to the time protocol given in the text. Concentrations of aldehydes in the headspace using NO^+ precursor are shown in parts-per-billion by volume (ppbv). Error bars indicate the standard deviation of the concentrations obtained in each of the 60 one-second integration intervals.

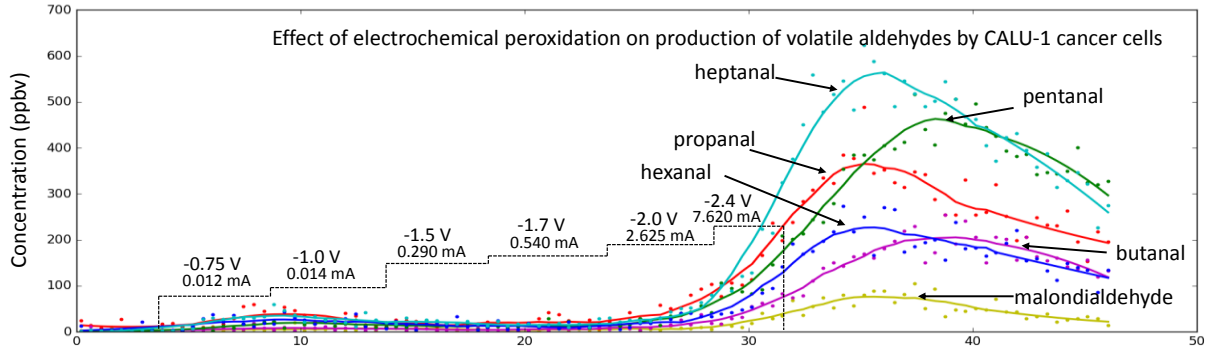

Figure 5. SIFT-MS real time quantification of volatile aldehydes in the headspace of CALU-1 cells during indirect electrochemical peroxidation. Different potentials from -0.75 V to -2.4 V were applied every 5 minutes for a 30 minutes period as indicated by the staircase line. Concentrations of aldehydes in the headspace are shown in ppbv.