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Direct analysis of *in vitro* grown microorganisms and mammalian cells by ambient mass spectrometry

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Mass spectrometry (MS) is a firmly established method for *in vitro* cell studies with numerous applications documented in the literature. Considerable sample pretreatment is normally required for MS cell analysis assisted by classic ionization techniques including electrospray ionization (ESI), electron-impact ionization (EI), matrix-assisted laser desorption/ionization (MALDI), chemical ionization (CI), *etc.* The speed and throughput of mass spectrometric analysis have been dramatically improved with the emergence of ambient sampling methods, requiring little-to-no sample preparation for virtually any type of biological samples. Direct chemical sampling makes MS analysis simpler, faster and less invasive to living objects. To date, considerable experimental evidence has been accumulated that strongly indicates the broad applicability of ambient mass spectrometry (AMS) in different areas of cell research. Particular areas include early detection and rapid classification of pathogenic bacteria, authentication of cancer cell lines, drug/therapy development, biomarker discovery, clinical diagnosis, as well as the mechanistic studies of cellular metabolism and cell–host interactions. In this review, we summarize recent AMS studies on *in vitro* grown cells of microorganisms and mammals and systematically describe current analytical strategies, technologies and achieved results. The perspective of ambient mass spectrometry for this particular field is also discussed.

Introduction

Studies of isolated microorganisms and animal cells grown in the laboratory under controlled conditions *in vitro* are indispensable in molecular biology and medicine. Cell cultures serve as models to develop efficient diagnostics and therapy of diseases as well as to improve our understanding of cellular metabolism.[1–3] Conventional biochemical assays to characterize cell cultures commonly suffer from the low sensitivity and specificity of analysis, which makes them time-consuming and prone to errors.[4,5] Alternatively, cells can be examined by a wide selection of analytical methods, including chemical sensing, optical spectroscopy, "electronic noses", *etc.*[6–13] These methods allow reliable distinction between cell cultures of different origin or between cells grown under different physiological conditions, but they often lack the required chemical specificity of analysis. Beneficial to the aforementioned methods, mass spectrometry (MS) combines ultrasensitive and high-mass-resolution detection with excellent molecular specificity, which greatly enhances the speed and information capacity of analysis. MS has become one of the central techniques in cellular proteomics and metabolomics for the identification of biomarkers, drug discovery and metabolic pathway analysis.[14–20] However, common MS-based workflows are rather complicated and include many laborious steps such as cell lysis, enzymatic digestion, purification, labeling, as well as 2D gel, ultra-pressure liquid chromatography (UPLC) or gas chromatography (GC) separation. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for the rapid and simple fingerprinting of bacteria is by far the most popular *in vitro* application of MS.[21–25]

Ambient sampling gave rise to the entire new field of ambient mass spectrometry (AMS).[26–31] In AMS, samples are analyzed without pretreatment directly from their native environment. Ambient methods found broad application in biological studies because they allow direct, rapid and simple MS analysis of complex matrices, *e.g.*, human urine or blood.[32,33] AMS imaging of biological tissues in open air without sample pre-treatment is a rapidly developing field.[34–36] Many ambient ion sources are non- or low-invasive and are therefore particularly suitable for *in vivo* studies of living objects, such as bacteria,[37–39] plants,[36,37,40–42] animals[41,43,44] or humans.[45–47] Noninvasive real-time AMS analysis was also applied for the intraoperative molecular diagnosis of tumors and tissue identification.[48–50] *In vivo* analyses of biological systems by AMS are summarized in recent review papers.[51,52]

AMS analysis of *in vitro* grown cell cultures allows investigation of cellular metabolism with greater simplicity

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compared to the traditional methods. Molecular fingerprints of isolated bacterial and human cells offer a benchmark for the identification and classification of corresponding species. Over recent years, a variety of AMS approaches have been developed to characterize *in vitro* grown cells of microorganisms and mammals with little-to-no sample preparation, extraction, or pre-separation (Tables 1 and 2). The data accumulated to date indicate the broad applicability of AMS in different areas of cell research, such as classification of isolated bacteria, authentication of cancer cell lines, drug/therapy development, biomarker discovery, clinical diagnosis, as well as the mechanistic studies of cellular metabolism and cell–host interactions. In this review we summarize the recent studies and systematically describe current analytical strategies, technologies and the achieved results. Finally, the emerging trend for the analysis of single cells with ambient MS is highlighted, and current limitations are discussed.

Analysis of volatile metabolites

Both microorganisms and mammalian cells are known to have characteristic smells due to the specific profile of volatile organic compounds (VOCs) released as part of their metabolism.[53–55] Hence is the sustained interest to the analysis of VOCs released by cell cultures.[7–12,53,56–72] Unique VOC fingerprint can be used as a biomarker for the identification of microorganisms or animal cell lines. Identification by fingerprinting is usually much more accurate and reliable compared to the identification based on just one biomarker metabolite.[73,74] The difference in fingerprints can be visualized by statistical analysis such as PCA. However, chemical identification of biomarker signals is essential to validate their use in clinical studies. Because VOC sampling inflicts minimal alterations to the culturing conditions, it can be a convenient approach to monitor metabolic changes continuously in real time,[75–78] *e.g.*, in response to antibiotic/drug treatment, nutrient deprivation, light, or other

Table 1 MS methods with ambient sampling applied in *in vitro* cell studies

		VOCs		Non-VOCs		
Method	Principle	Microbes	Mammal cells	Microbes	Mammal cells	Highlights
SIFT-MS	Chemical ionization (CI) of vapors by H_3O^+, NO^+ and $O_2^{+\cdot}$	68, 69, 77, 98–101 and 106	65, 110–112			Quantitative detection of hydrogen cyanide from *Pseudomonas aeruginosa*. *In situ* profiling of competition between various species in real time. Studies of cancer cell metabolism *in vitro*.
PTR-MS	CI of vapors by H_3O^+	61, 66, 75, 76 and 93	78 and 113			Measuring metabolic response to external factors in real time. Monitoring fermentation process. *In vitro* identification of lung cancer cells based on VOC fingerprint.
IMR-MS	CI of vapors by Kr^+, Hg^+ or Xe^+	64 and 67				Broad molecular range of detection. Rapid identification of Gram-positive and Gram-negative bacteria by VOC fingerprints.
SESI-MS	Ionization of vapors by ESI	63, 82 and 109				Comparison between *in vitro* and *in vivo* VOC fingerprints of pathogenic species. Easily implemented on any ESI-MS instrument.
DESI-MS	Desorption/ionization by pneumatic ESI			115, 118, 119, 129 and 130		Tolerate high salt concentration in the culture medium. Direct spatial profiling of chemical gradients in a growing culture. Single-cell analysis. Broad molecular range.
DART-MS	Desorption/ionization by gas plasma			121 and 132		Rapid non-targeted screening for the identification of cellular metabolites.
LTP-MS	Desorption/ionization by gas plasma			117		Rapid identification of bacterial strains on the species and subspecies level. Low-temperature desorption. High stability.
REIMS	Desorption/ionization by RF current			116		Identification of clinical isolates directly from the agar plate without sample prep. Rich chemical capacity.
LAESI-MS	Desorption by IR irradiation; Ionization by ESI			122	120 and 133	Observation of proteins. Analysis of small cell populations down to single-cell. *In vitro* metabolism of virus-infected human cells. Suitable for the direct analyses of cells cultured in aqueous media.

Table 2 Microorganisms, cell lines and analyte chemicals in *in vitro* cell studies by ambient MS

Microbes	*Acinetobacter*,[67] *Anabaena*,[122] *Bacillus*,[117,118] *Citrobacter*,[116] *Coxiella*,[132] *Enterobacter*,[67] *Enterococcus*,[64] *Escherichia coli*,[67,77,93,109,116–118,132] *Klebsiella*,[67,116] *Malbranchea*,[121] *Mycobacterium*,[75] *Proteus*,[67,116] *Pseudomonas*,[67,69,82,101,106,116] *Salmonella*,[109,117,118] *Serratia*,[67,77,116] *Staphylococcus*,[64,66,82,109,116–118] *Streptococcus*,[116,132] *Synechococcus*,[115] *Thalassiosira*[76]
Cell lines	293T (kidney epitelium),[133] 35FL121 Tel+ (lung fibroblast),[111] A-549 (lung epitelium),[78,113] BCBL-1 (B-lymphocytes),[120] BEAS-2B (bronchial epithelium),[113] BJAB (B-lymphocytes),[120] C81 (T-lymphocytes),[133] CALU-1 (lung carcinoma),[111,112] CEM (T-lymphocytes),[133] EPLC-M1 (lung carcinoma),[113] H9 (T-lymphocytes),[133] HepG2 (hepatocellular carcinoma),[65] MSC (mesenchymal stem cells),[65] NL20 (lung epithelium),[111,112] TERTR-PE1 (retinal epitelium)[78,113]
Volatile analytes	Acetaldehyde,[65,75,77,93,110–113] acetic acid,[77] acetoin,[77] acetone,[75,77] acetophenone,[106] ammonia,[77] carbon dioxide,[110] dimethyl sulfide,[65,76] ethanol,[75,77] hydrogen cyanide,[69,101] isoprene,[76] methyl-butanone,[106] methyl thioacetate,[106] methyl thiobutanoate,[106] propanol,[77] fatty acids[116]
Non-volatile analytes	Alkaloids,[121] amino acids,[122,133] dopamine,[133] fatty acid esters,[117,132] glucosylglycerol,[115] homovallinic acid,[133] lipids,[115,116,118,120,122,133] nucleotides,[122,133] phosphatidic acids,[116] phosphatidylethanolamine,[116] proteins,[120,122] sugars[115,122]

environmental factors. Particularly intriguing is the possibility of direct comparison between *in vitro* studies on isolated cell cultures and *in vivo* measurements of VOC metabolites,[79–82] *e.g.*, released with breath or emanated from skin.[45,46] Such integrated approach can be used for diagnostics purpose, *e.g.*, identification of pathogens or cancer. Finally, chemical identification of VOC metabolites is important to develop simple, inexpensive and reliable VOC sensor arrays for the early targeted detection of pathogen biomarkers in blood cultures and authentication of animal cell lines.[83–85]

Delicate approach is required in order to interrogate *in vitro* grown cells without harmful effects. The major figures of merit associated with sampling volatile metabolites include non-invasiveness, ease of practical implementation and toxicological safety. Over decades, the combination of gas chromatography with electron-impact ionization mass spectrometry (GC-MS) has been essentially the only available tool to probe the headspace of cell cultures by MS.[86,87] However, this mode of VOC analysis is time-consuming and cannot be implemented in real time. Furthermore, sensitive and surface-active metabolites can undergo chemical transformation upon sample collection and storage.[88]

The situation started to change with the invention of selected-ion flow-tube mass spectrometry (SIFT-MS)[88–90] and proton-transfer reaction mass spectrometry (PTR-MS).[91–93] These methods allow real-time profiling of VOC metabolites without the need for chromatographic separation, sample collection and regular calibration. Beneficially, SIFT-MS and PTR-MS were developed into commercial products. Commercial instruments offer accurate quantitative detection of volatile compounds, which is an essential requirement if these MS techniques are to be used in clinical diagnosis. Both SIFT-MS and PTR-MS have very similar principle of operation.[94] In SIFT-MS, primary reagent ions are isolated from microwave air plasma using a quadrupole mass filter and transferred into the reaction flow tube with helium gas flow at a pressure of ~1 Torr. Most common reagent ions in SIFT-MS include H_3O^+, NO^+ and O_2^+ because they can efficiently ionize many gas molecules but have little reactivity toward the major air components, *i.e.* nitrogen, oxygen, *etc.* Sample vapor is introduced into the flow tube *via* a heated sampling tube. Secondary analyte ions are produced by chemical ionization (CI) with the primary reagent ions and are transferred into analytical quadrupole for mass detection. PTR-MS is different from SIFT-MS in that it typically employs water vapor to produce primary H_3O^+ ions and that primary ions are not pre-isolated in a quadruple mass filter. Later, another CI-based method called ion-molecule reaction mass spectrometry (IMR-MS) has been introduced for the direct analysis of VOCs.[95,96] IMR-MS uses chemical ionization of volatile molecules by Kr^+, Hg^+ or Xe^+ primary ions and thereby extends the molecular range of detection up to the ionization energy of Kr (14 eV). Secondary ions in IMR-MS are mass analyzed using quadrupole ion optics, similar to PTR-MS and SIFT-MS. The method features no or only minimal fragmentation.[95] Most recently, secondary electrospray ionization mass spectrometry (SESI-MS) has been developed for the analysis of vapors.[46,97] In SESI-MS, vapor molecules are ionized by electrospray droplets of organic solvent mixture, *e.g.*, water/methanol in front of MS inlet. Similar to PTR-MS, ionization of analyte molecules in SESI-MS is usually achieved by protonation. However, ionization in SESI-MS occurs in the ambient environment in front of a mass spectrometer rather than in a reaction chamber, like in SIFT-MS, PTR-MS or IMR-MS. Therefore, SESI-MS can be implemented on any type of MS instrument with atmospheric pressure ionization interface, which makes it more versatile for VOC studies. Provided the appropriate type of mass spectrometer has been adapted for SESI, selected peaks can be fragmented for chemical identification, which is another distinct advantage of the method. A number of other AMS approaches are available for VOC analysis but, to our knowledge, those have not yet been applied to bacterial and mammal cell cultures. Note that PTR-MS and SIFT-MS are sometimes considered separately from other AMS methods, because they require accordingly designed mass spectrometers and are difficult to interface with most popular instruments.[52] Nevertheless, sampling in PTR-MS and SIFT-MS is implemented in the ambient environment, allowing direct real-time measurements without sample preparation. Hence, we included PTR-MS and SIFT-MS applications in the scope of this review.

Continuous, real-time monitoring of VOC metabolites from the headspace of growing cultures is achieved with dedicated sampling interfaces, *e.g.*, as illustrated in Fig. 1a. The cell-containing medium is incubated in a sealed fermenter or other

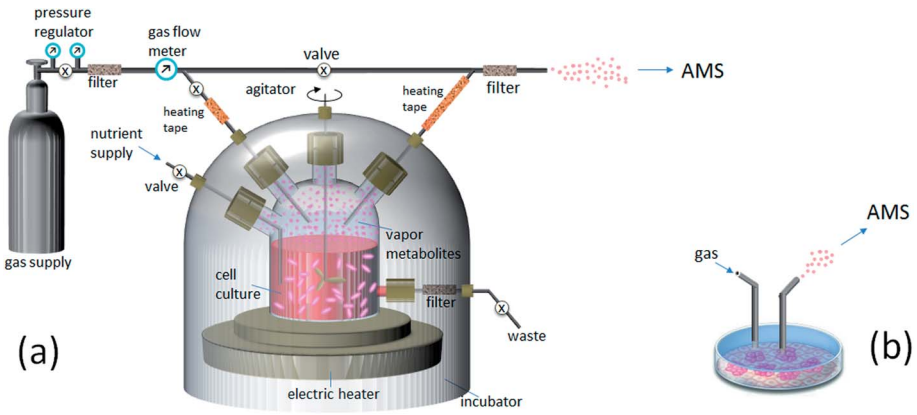

Fig. 1 Sampling VOC metabolites from a growing cell culture for ambient mass spectrometry (AMS): (a) from a fermenter; (b) from a plate.

container vented by a gas flow with controlled composition chosen to best mimic in vivo environment for specific cells. For example, for the growth of lung cancer lines a mixture of clean air (95%) and CO_2 (5%) is normally used, which corresponds to the composition of exhaled human breath. Particle filters prevent possible chemical contamination of the media by impurities originated from the gas supply or desorbed material from transfer tubing. The cells are grown under controlled temperature (normally 37 °C), nutrient supply and disposal. Agitation of media volume is often applied to prevent cell coagulation and minimize adhesion to the fermenter walls. VOC metabolites are continuously transferred with the gas flow out of the headspace volume into the outlet tubing line for ionization and MS detection. Alternatively, if the real-time profile of headspace VOCs is not necessary, conventionally grown cell culture can be removed out from the incubation chamber for a rapid noninvasive VOC inspection by AMS and then returned back with minimal adverse consequences (Fig. 1b). Such format of sampling is practical for slowly growing mammalian cells, which are prone to contamination, or/and when rapid MS fingerprinting of a cell culture is demanded.

1. Analysis of microbial cultures

SIFT-MS is the most widely applied AMS method for the analysis of microbial VOCs.[20,69,77,89,90,98–106] Using SIFT-MS, Carroll *et al.* studied VOCs emitted by *Pseudomonas aeruginosa* (PA) cultures derived from the sputum samples of 21 patients with cystic fibrosis (CF).[69] The cultures were grown on blood agar with *Pseudomonas*-selective media for 48 h at 37 °C inside sealed plastic bags. Among many other VOCs, hydrogen cyanide (HCN) was revealed at the ppm level (Fig. 2). Even though fragmentation capabilities of SIFT-MS are limited, protonated H_2CN^+ cation was unambiguously identified because the three other possible ions at *m*/*z* 28, *i.e.*, N_2^+, CO^+ and $C_2H_4^+$, could not be generated under the same experimental conditions. In the

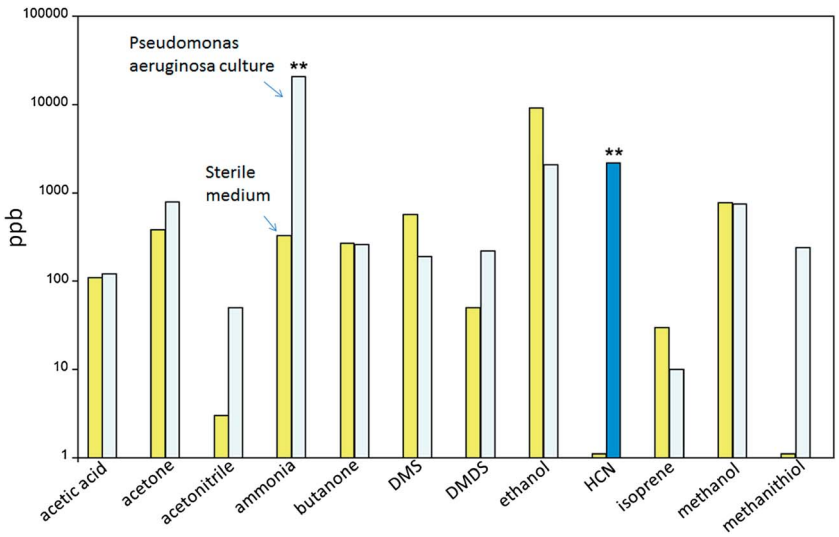

Fig. 2 The average concentrations of some VOCs determined by SIFT-MS in the headspace of sterile media (left, yellow bars) and *Pseudomonas aeruginosa* plate cultures (right, blue bars). The statistical significance of the increase in ammonia and HCN is below 0.01 as indicated by **. Adapted from ref. 105.

absence of spectral interferences, HCN was detected by SIFT-MS with high sensitivity and quantified with high accuracy. This study promoted extensive research on the detection of HCN in PA cultures[101] and in the breath of patients with PA infection.[102–104] As a result, breath HCN was established as a reliable biomarker for PA infection in humans. The timeline for this research is given in a recent review paper by Smith *et al.*[105] Recently, Shestivska *et al.* combined VOCs with particularly high specificity to PA, such as 3-methyl-butanone, acetophenone, methylthioacetate and methyl thiobutanoate, in a reference SIFT-MS fingerprint for *in vivo* identification of various PA strains with higher specificity compared to a single biomarker.[106] Interestingly, quantitative SIFT-MS analysis revealed that the production rates of volatile metabolites, including HCN, from genotypically diverse PA strains vary by two orders of magnitude under the same culturing conditions.[101,106] Similar variability was observed for liquid and solid media. Besides the mere identification purposes, quantitative SIFT-MS was also demonstrated as a valuable tool to probe mechanistic aspects of bacterial metabolism. Sovova *et al.* monitored the real-time population dynamics of three different bacterial species, *Serratia rubidaea* (R), *Serratia marcescens* (F) and *Escherichia coli* (Ec), growing in liquid media.[77] The concentrations of VOC metabolites (ammonia, ethanol, acetaldehyde, propanol, acetoin, acetone and acetic acid) were measured in the headspace of the individual species and of their mixtures continuously for 24 h periods. The three bacterial species were found to interact with each other in a competitive fashion in a way similar to the game "rock-paper-scissors" (R-Ec-F).[77]

As a tool for quantitative detection of VOCs in real-time, PTR-MS has similar capabilities to SIFT-MS. PTR-MS is widely established in environmental and food chemistry research, but its biomedical applications are fairly recent. Using PTR-MS, Crespo *et al.* monitored the headspace of growing *Mycobacterium smegmatis* cultures in real time in response to the addition of antibiotics ciprofloxacin and gentamicin.[75] Following the emission patterns of the mycobacteria over time allowed detection of volatile markers specific for the bacterial response, *e.g.*, acetaldehyde (*m*/*z* 45). Antibiotic-specific response was evident already 3 h after the treatment and varied between isolates with different resistance phenotypes. The early observation of metabolic response indicated the high sensitivity of PTR-MS diagnostics compared to traditional biochemical assays. The high signal stability in PTR-MS allows observations of VOCs emitted by microorganisms over long time intervals, and therefore even slow metabolic changes can be revealed in real time. Kameyama *et al.* used PTR-MS to continuously monitor VOC emission from the marine diatom *Thalassiosira pseudonana* in an axenic batch culture system under a 13 : 11 h light–dark cycle.[76] The authors found that the intensity of signal at *m*/*z* 69, tentatively assigned to isoprene, rapidly changed upon the light–dark and dark–light transitions, suggesting a crucial role of light in the production of this metabolite. In contrast, the intensity of signal at *m*/*z* 63, tentatively assigned to dimethyl sulfide (DMS), did not reveal clear diurnal variation during the early incubation period. The observed light response for DMS was found similar to that in senescent cells rather than vegetative cells. Therefore, DMS production in the studied *Thalassiosira pseudonana* culture was attributed to cell aging and/or cell death. The authors suggested that, along with the other microbial processes, aging or death of phytoplankton cells could mediate an important role in the regulation of DMS production in natural waters.[76] Recently, Luchner *et al.* developed an interface to analyze the headspace of bioreactors with PTR-MS. To proof-test the platform, recombinant *E. coli* strain was cultured in a 20 L bioreactor in a fed-batch mode for 20 h, and the emitted VOCs were monitored by PTR-MS in real time (Fig. 3). Highly reproducible PTR-MS profiles were recorded during three identical cultivations for chemicals in the broad concentration range of *ca.* 10–10 000 ppb, indicating the high potential of PTR-MS to reliably monitor large-scale fermentation processes.[93]

Owing to its high sensitivity and softness, IMR-MS is widely applied to analyze VOCs in exhaled breath.[107,108] Recently, a project has been initiated aiming to evaluate the suitability of VOC fingerprinting by IMR-MS as a diagnostic tool for bacterial infections. In a pilot study, Dolch *et al.* analyzed VOCs from selected Gram-positive bacteria that are frequently isolated in blood culture samples, namely, *Enterococcus faecalis,*

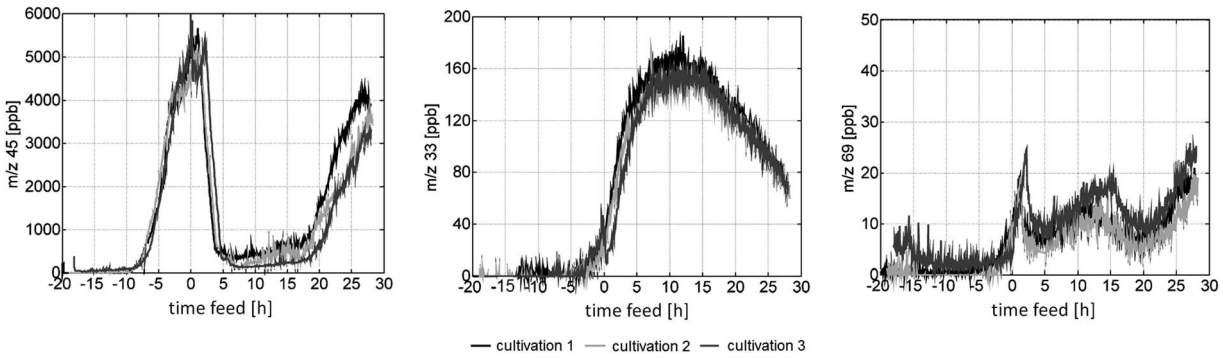

Fig. 3 Highly-reproducible time profiles of typical PTR-MS signals measured in a broad intensity range during three recombinant *E. coli* fed-batch cultivations. The signals at *m*/*z* 45, *m*/*z* 33 and *m*/*z* 69 were tentatively assigned to acetaldehyde, methanol and isoprene, accordingly. Adapted from ref. 93.

Enterococcus faecium, Staphylococcus aureus, and Staphylococcus epidermidis.[64] Using IMR-MS, characteristic VOC profile could be detected for *E. faecalis* already 8 h after incubation. After 24 h of incubation, all the studied species could be differentiated by hierarchic clustering and principal component analysis (PCA) of generated MS fingerprints. The focus of the study was to achieve rapid differentiation of bacterial species with the possibility of workflow automation, while the identification of biomarker signals was not attempted. The study was successfully extended to differentiation of Gram-negative bacteria, *Acinetobacter baumannii*, *Enterobacter cloacae*, *E. coli*, *Klebsiella oxytoca*, *P. aeruginosa*, *Proteus vulgaris* and *Serratia marcescens*, with similar speed of identification by IMR-MS.[67]

SESI-MS is the most recent AMS technique for vapor analysis. Based on characteristic VOC fingerprints recorded by SESI-MS, Zhu *et al.* were able to detect and separate a group of 11 pathogenic *E. coli* strains from two major foodborne bacteria, *Staphylococcus aureus* and *Salmonella Typhimurium* in three food modeling media.[109] Heatmap analysis revealed six core peaks (m/z of 65, 91, 92, 117, 118 and 119) present at a similar intensity in all 11 *E. coil* strains. These peaks were proposed to be conserved VOC biomarkers for *E. coil* species. Notable signals were observed just 4 h after incubation, indicating the high sensitivity of approach. In another study from the same group, Zhu *et al.* investigated volatile metabolites of *P. aeruginosa* (strains PAO1, FRD1) and *S. aureus* (RN450) cultured *in vitro* by SESI-MS to identify characteristic spectral patterns. *In vitro* studies were followed by SESI-MS breath analysis of infected mice to collect MS fingerprints of corresponding bacteria species *in vivo*. Interestingly, it was found that only 25–34% of peaks were shared between the *in vitro* and *in vivo* SESI-MS fingerprints. This significant difference can be attributed to a combination of factors including altered bacterial metabolism inside a host, generation of new metabolites specific to pathogen–host interaction, and lower sensitivity of SESI-MS to detect VOCs from breath compared to cell culture.[82] The study underscored the challenges of *in vitro* models to predict *in vivo* responses.

2. Analysis of mammalian cell lines

Compared to microorganisms, VOCs of mammalian cells are less studied. Mammalian cell cultures grow much slower, are more difficult to handle, prone to contamination, and more expensive than bacteria. Nevertheless, a number of recent studies indicate that the sensitivity of SIFT-MS and PTR-MS is sufficient to directly characterize VOCs emitted by cell lines. The level of acetaldehyde (AA) emission has been proposed as a potential indicator to differentiate normal and cancer cells. Using quantitative SIFT-MS analysis, Smith and coworkers explored the release of AA (m/z 45) and other volatile metabolites in the headspace of CALU-1 lung cancer cells, NL20 normal lung epithelial cells and 35FL121 Tel+ telomerase positive lung fibroblast cells grown *in vitro*.[110,111] Cultures contained 50–80 $\times$ 10^6 cells and were incubated for 16 h at 37 °C. Interestingly, notable amounts of AA were observed from the CALU-1 and NL20 cells but not from 35FL121 Tel+. It was proposed that the concentration of headspace AA in a cell culture can reflect the activity of aldehyde dehydrogenase (ALDH). In line with this hypothesis, the same group later found that the intensity of SIFT-MS signal for AA measured from hepG2 hepatocellular carcinoma cells and primary bone marrow-derived mesenchymal stem cells (hMSCs) could be regulated by the addition of ALDH inhibitors to the growing culture.[65] Most recently, the same group attempted SIFT-MS analysis to monitor the release of AA by CALU-1 and NL20 lung cells seeded in 3D collagen hydrogels, which are more physiologically relevant models compared to 2D.[112] The data showed that the amount of AA released by the both cell types grown in a 3D scaffolds is higher than for the same cells grown in 2D models. AA from the headspace of lung cancer cells could be measured even at a cell concentration as low as 105 cells per hydrogel. The differential of AA release could be, depending on the cell concentration, more than 3 fold higher for cancer cells when compared to non-malignant lung cells. Using PTR-MS, Brunner *et al.* analyzed VOCs emitted by four *in vitro* cultured human cells: lung epithelium cells A-549, retinal pigment epithelium cells hTERTR-PE1, squamous lung carcinoma cells EPLC and immortalized human bronchial epithelial cells BEAS2B.[113] The VOCs in the headspace of the cell cultures were sampled either online by continuous transfer directly from the culture flask or by 12 h collection in PTFE bags connected to the flask. The pure media were analyzed in the same way as the corresponding cell cultures in order to provide a reference. By applying multivariate statistical analysis using 42 selected marker VOCs, it was possible to clearly separate the cancerous and non-cancerous cell lines from each other. The authors also observed substantial consumption of headspace AA (m/z 45) by the cancerous cell lines but not by the non-cancerous cells. However, given the earlier observation of AA from some non-cancerous cells by SIFT-MS,[111] the specificity of AA level to cancer cells remains unclear. The limited experimental data available at present should be substantially extended to address the potential of AA and other VOCs as a volatile biomarker of tumor cells.

Analysis of nonvolatile chemicals

The concentration and diversity of VOCs released by a growing culture represent a considerable challenge for the direct identification and characterization of cells by AMS with high chemical specificity. Alternatively, sampling of nonvolatile chemicals from the cell surface and/or cell interior offers much richer chemical information of analysis, but this mode of sampling is invasive. Traditional MS methods for the analysis of cellular proteins and metabolites include ESI-MS and MALDI-MS in combination with 2D gel and/or liquid chromatography. These methods allow deep coverage of cell proteome and metabolome but involve laborious sample preparation, complex and time-consuming workflow as well as the high cost of operation. A simpler workflow is used for the analysis of membrane lipids by MALDI-MS, but sample preparation, *e.g.*, matrix deposition and cell washing, is still required. Furthermore, all the above analyses cannot be done in real time.

Ambient ion sources allow sampling of nonvolatile cell chemicals including metabolites, lipids and proteins with little-to-no sample preparation. Because ambient methods tolerate very high salt concentrations and other matrix effects,[33,114] sampling can be performed directly from a growing culture without any pretreatment (Fig. 4a).[115,116] For the higher specificity and sensitivity of detection, cells are commonly separated from the medium prior to analysis.[117,118] Harvested cells are washed in aqueous buffer and then subjected to desorption/ionization, *e.g.*, from a glass slide (Fig. 4b). Note that the cell integrity is normally preserved during these steps, so the intact species are eventually sampled. The analysis of intact cells is beneficial for the studies on cellular metabolism and allows faster and simpler workflow compared to the methods relying on chemical extraction. However, the preserved viability of cells during the analysis can also represent a problem, necessitating careful experimental design to prevent aerosolization of pathogenic species into the ambient air. Possible solutions to this problem include the use of enclosed sampling interfaces as well as long-term drying (>1 h at room temperature) of cells on a sample holder prior to the analysis for better adhesion.[119] Because chemical desorption in AMS results in gradual cell degradation, mass spectral response is prone to alterations during the analysis. Rastering the position of a probe can partially account for this problem, but the signal stability in this case is strongly affected by spatial inhomogeneity of the analyzed cell colony. This makes real-time profiling of metabolic processes by the desorption-based AMS methods rather complicated. Instead, the most promising applications for these methods appear to be the rapid identification of isolated species[116,118,120] and untargeted metabolite screening.[120–122]

The ionization method for the direct cell sampling should meet two major criteria. First, the number of interrogated cells should be negligibly low compared to the entire cell population in the culture, in order to minimize adverse effects to the culture viability during the analysis. Second, rather harsh desorption conditions are needed in order to efficiently break the walls of exposed cells and extract internal chemicals. Preferred methods employ charged droplets, plasma or local heating as a source of ionization. Desorption electrospray ionization (DESI)[123] is by far the most popular AMS method for surface analysis and has been developed into commercial product. In DESI-MS, chemical extraction and ionization of analytes on the surface are achieved by means of pneumatic electrospray with a spatial resolution down to 35 μm.[124] When applied to cell cultures, DESI plume primarily desorbs chemicals from the outer cell layers together with excreted metabolites. The penetration depth of ionization depends on cell rigidity. Thus, owing to the thick walls Gram-positive bacteria can withstand the impinging sprayed droplets fairly well, preventing deep penetration of the ionization.[118] DESI-MS spans a broad molecular range, from small metabolites to proteins. Ionization of polar molecules is usually achieved *via* proton transfer. Nonpolar molecules can be charged *via* complex formation with ionic compounds added to DESI spray, *e.g.*, metal cations. In plasma-based ionization methods, direct analysis in real time mass spectrometry (DART-MS)[125] and low temperature plasma probe mass spectrometry (LTP-MS),[47] ambient beam of excited-state atoms and ions, typically nitrogen and helium, are used to desorb and ionize molecules from the surface of a sample. Plasma-based methods allow sensitive detection for a wide range of small molecular analytes, but larger molecules such as proteins normally remain invisible. Beneficially, ionization with gas plasma minimizes adverse effects to the culture growth and reduces operational costs associated with the use of organic solvents. DART is by far the most popular AMS plasma ion source and has been developed into commercial product that can be installed on most mass spectrometers with atmospheric pressure interface. The key advantage for LTP is the use of dielectric barrier discharge to induce the low-energy plasma through use of a specially designed electrode configuration. The low-energy plasma allows experiments to be performed without damage to the cell colony or underlying substrate due to electrical shock or perceptible heating. Finally, in laser ablation electrospray ionization mass spectrometry (LAESI-MS)[42] and rapid evaporative ionization mass spectrometry (REIMS)[50] chemical desorption from the sample surface is achieved by strong locally-confined heating. In LAESI-MS local heating is achieved using IR laser irradiation (LAESI-MS). The plume of desorbed molecules and clusters is intercepted by electrospray for ionization. ESI ionization allows the observation of small polar metabolites and lipids as well as intact proteins. With the help of etched optical fibers, laser irradiation can be focused down to *ca.* 20 μm, which makes

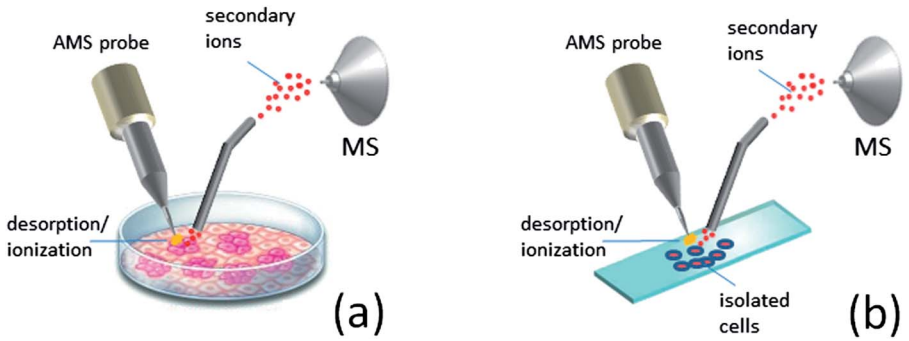

Fig. 4 Ambient sampling of nonvolatile cellular chemicals: (a) directly from the growth medium; (b) from isolated cells deposited on a glass slide.

LAESI-MS particularly suitable for the chemical analysis of very small cell populations or even single cells.[126–128] REIMS was originally introduced for real-time, *in vivo* analysis of biological tissues in the surgical environment.[49,50] Recently, REIMS methodology was extended for the analysis of cell cultures. Bacterial biomass is rapidly heated by the application of RF electrical current. Thermally induced disruption of cells leads to the production of an aerosol containing gas-phase ions of metabolites and structural lipids. The aerosol is directly introduced into a mass spectrometer for on-line chemical analysis.

1. Analysis of microbial cultures

The potential of DESI-MS for the analysis of microbial cultures has been demonstrated in several studies.[118,119,129,130] Recently, Zhang *et al.* used DESI-MS to characterize lipid composition of four different bacterial species, *Bacillus subtilis, Staphylococcus aureus, E. coli* and several *Salmonella enterica* strains.[118] Cells were harvested from growing cultures, suspended in 70% ethanol and then evenly deposited on a microscope glass slide (*ca.* 3000 cells per slide) for direct mass analysis without chemical extraction or other sample preparation. Many of the bacterial species could be distinguished visually based on the distribution of lipid signals in DESI-MS spectra (Fig. 5a). Lipids representative of several major classes, including phosphatidylethanolamines (PE), phosphatidylglycerols (PG) and lysyl-phosphatidylglycerols (LPG), were characterized by tandem MS analysis. PCA analysis of the generated DESI-MS fingerprints clearly differentiated all the four bacterial species studied and several (although not all) of 13 *Salmonella* strains (Fig. 5b).[118] Identification of bacteria by DESI-MS fingerprinting bears great similarity to the established workflow for the identification of bacterial cultures by MALDI-MS.[23] Beneficially, DESI-MS obviates matrix deposition and sample loading steps, which makes the analysis simpler and faster.

Using a variation of DESI called nanospray desorption electrospray ionization (nano-DESI),[131] Lanekoff *et al.* have recently demonstrated that, despite the high salt content of the agar (*ca.* 350 mM), bacterial cultures can be analyzed directly from agar plates, obviating cell harvesting and washing.[115] Spatial analysis

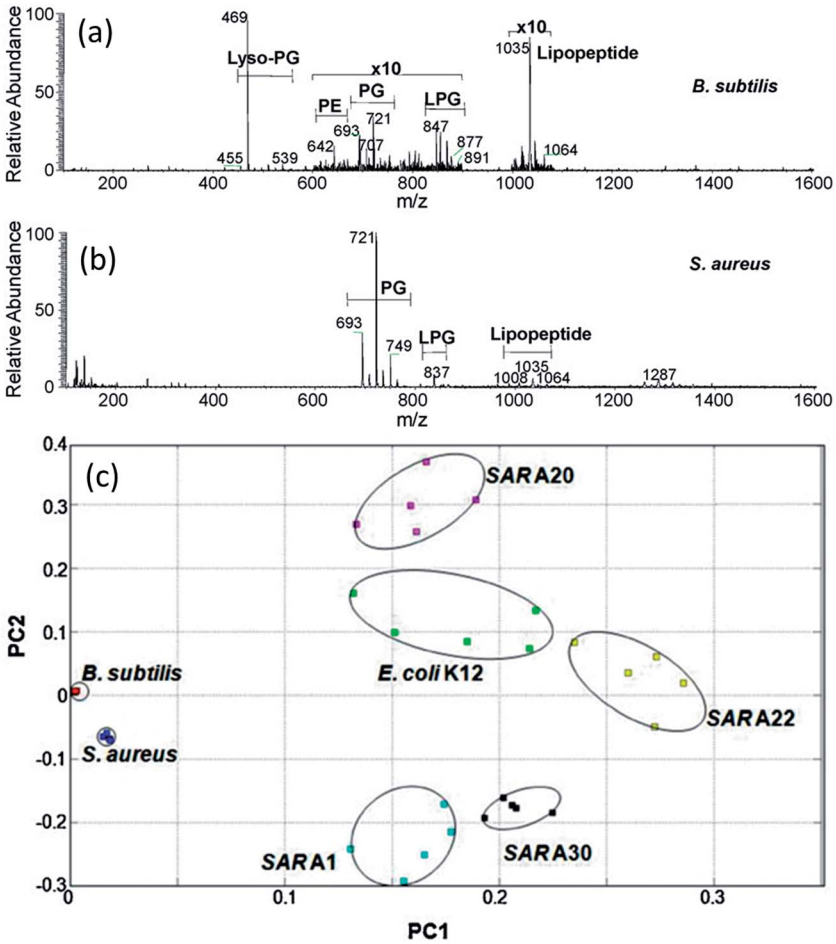

Fig. 5 Analysis of bacterial cells with DESI-MS. (a and b): Negative ion mode DESI mass spectra of gram-positive bacteria with major compound categories shown. (a): *B. subtilis* DESI spectrum with strong lyso-PG peak at *m/z* 469, regions *m/z* 600–900 and 1000–1100 expanded by 10 times showing PE, PG, LPG and a relatively strong C15 surfactin lipopeptide signal at *m/z* 1035. (b): *S. aureus* DESI spectrum with strong PG peaks in the *m/z* 700 range and some LPG and lipopeptides. (c): Score plot of PCA results for the negative ion mode DESI mass spectra of *B. subtilis*, *S. aureus*, *E. coli* K12, and four *Salmonella* strains (SARA1, SAR A20, SAR A22 and SAR A30). Adapted from ref. 118.

of *Synechococcus* sp. PCC 7002 colonies by nano-DESI-MS revealed the occurrence of chemical gradients due to bacterial metabolism. Thus, a majority of lipids and metabolites were localized on the colony while sucrose and glucosylglycerol, an osmoprotective compound produced by cyanobacteria, were secreted onto the agar. The chemical gradients of sucrose and glucosylglycerol on agar plate were found to depend on the age of the colony. Tandem nano-DESI-MS analysis revealed several glycolipids that have not been previously reported by conventional methods based on chemical extraction.[115] AMS fingerprinting of cellular metabolites directly from a growing culture opens new possibilities for the rapid identification of microorganisms and studies of cellular metabolism in real-time.

Plasma ion sources do not require any spray solvent and are usually distinguished by the high stability, simplicity and low cost of operation. These figures of merit are particularly important for the screening of large sample arrays such as bacterial libraries. Pierce *et al.* used DART-MS to fingerprint fatty acid methyl esters (FAMEs) in isolated bacterial cells (*Streptococcus pyogenes*, *E. coli*, *Coxiella burnetii*).[132] Isolated cells were washed in TRIS-sucrose buffer and suspended in aqueous solution of tetramethylammonium hydroxide (TMAH) to induce thermal hydrolysis and methylation of bacterial lipids. A 4 mL aliquot of analyte suspension was sampled to DART-MS as a hanging droplet on the end of the capillary tube. FAME fingerprints of the studied bacterial species could be directly differentiated by visual inspection without the necessity of statistical analysis.[132] In a different study, Watts *et al.* used rapid DART-MS analysis to survey secondary metabolites in the hyphae of *Malbranchea graminicola* fungus grown on agar plates, without the need for any organic extraction.[121] Subsequently, milligram quantities of discovered metabolites were purified from the extracts of large scale liquid cultures *via* selected-ion monitoring with traditional LC-MS. The combination of DART-MS screening and targeted LC-MS provided a rapid and facile route to pinpoint molecules of interest and streamline their isolation. This approach revealed two new halogenated prenyl-indole alkaloid metabolites of *Malbranchea graminicola*, named (−)-spiromalbramide and (+)-isomalbrancheamide B. The authors also found that two new brominated analogues, (+)-malbrancheamide C and (+)-isomalbrancheamide C, were produced when the growth medium was enriched with bromine salts. This study indicated a promising niche for AMS to be used as a high-throughput screening tool to pinpoint potential biomarkers in cell cultures for subsequent characterization with methods having higher chemical specificity but slower scanning speed.

Using LTP-MS, Zhang *et al.* characterized the composition of fatty acid ethyl esters (FAEE) in *in vitro* grown bacteria *B. subtilis, S. aureus, E. coli* and *S. enterica*.[117] Bacterial samples from 70% ethanol solution were dried on a glass slide and then directly analyzed by LTP-MS. The sample preparation was similar to that in the earlier study by DESI-MS[118] to allow for the direct comparison of experimental data. PCA analysis of LTP-MS FAEE profiles successfully separated the different species (*B. subtilis, S. aureus, E. coli* and *S. enterica*) and 11 out of 13 *Salmonella* strains. Overall, LTP-MS method was found to yield more reproducible spectra and allowed better distinction between different bacterial strains. On the other hand, the information obtained by LTP-MS was largely limited to FAEEs in the range m/z 200–300, while DESI-MS also revealed fatty acids, phospholipids and lipopeptides in the range m/z 200–1500. This study served a nice illustration for the higher stability but narrower molecular range of plasma-based methods compared to solvent-spray methods such as DESI-MS or ESI-MS.

Using LAESI-MS analysis of vegetative *Anabaena* sp. PCC7120 cyanobacterial cells, Parsiegla *et al.* have recently demonstrated that, in addition to small metabolites and lipids, ambient sampling of bacterial colonies can also allow observation of proteins without the need of cell lysis.[122] *Ca.* 500–1000 washed intact cells were interrogated in a single LAESI-MS scan, resulting in over 30 metabolites and lipids (m/z < 900) tentatively identified based on accurate mass measurements, isotope distribution patterns, and literature search. The spectra also revealed phycocyanin (C-PC) and allophycocyanin (APC) (*ca.* 20 kDa), which belong to the family of phycobilisome proteins in the antenna complex, as well as the ratio of the α- and β-subunits within the C-PC and APC. Protein identification was based on the deconvolution of multiply charged ion peaks followed by mass comparison with proteins obtained from an annotated genome database. The high molecular range and spatial resolution (∼30 μm) of LAESI-MS are distinctive advantages for this method in cell studies.

Using REIMS, Strittmatter *et al.* have recently performed *in situ* characterization of nine bacterial species cultured on various growth media.[116] In REIMS, chemical desorption of cellular chemicals is achieved by applying RF electrical current to bacterial biomass. Thermally induced aerosol containing gas-phase molecules and ions is directly sampled into a mass spectrometer for on-line chemical analysis. Even though the produced aerosol is mostly constituted by neutral species, which are invisible by MS, REIMS spectra recorded in negative ion mode distinguished 100–400 spectral features in m/z range 150–2000. The high chemical capacity of the method is probably in part due to the high resolution and sensitivity of orbitrap MS detection used in that study. The dominant species in all the spectra were observed in m/z range 600–900 and corresponded to intact phospholipids. Correct classification of the studied bacterial species by the statistical analysis of MS data could be obtained when performing the analysis in m/z range 500–1900.[116] The broad molecular range, high chemical capacity, simplicity and speed of analysis make REIMS a competitive alternative to the widely used MALDI-MS approach in bacteria identification.[21,23,25]

2. Analysis of mammalian cell lines

Mammalian cell cultures are usually more difficult to handle compared to bacterial cultures, which limits their study in analytical MS laboratories. To our knowledge, LAESI-MS is by far the only successful ambient desorption method in this area of research. High throughput metabolite profiling by LAESI-MS combined with multivariate statistical analysis has been applied for the identification of virus-induced perturbations in

the biochemical processes of the host cells.[120,133] Sripadi *et al.* studied the metabolic effect of human T-lymphotropic virus (HTLV) on human cell lines.[133] Before LAESI-MS analysis, the cells were washed twice in phosphate buffered saline and pelleted by centrifugation to obtain *ca.* 10^6 cells per pellet. Pellets were loaded onto clean microscope slides for the LAESI-MS experiments. In a single-shot experiment, *ca.* 3000 cells were ablated. LAESI-MS spectra directly revealed the differences in the metabolite profiles specific to virus type (HTLV1 *vs.* HTLV3), Tax protein expression (Tax1 *vs.* Tax3) as well as the cell type (T lymphocytes *vs.* kidney epithelial cells). For example, glycerophosphocholine (PC) lipid components were dominant in the non-HTLV1 transformed cells, and PC(O-32 : 1) and PC(O-34 : 1) plasmalogens were displaced by PC(30 : 0) and PC(32 : 0) species in the HTLV1 transformed cells. In HTLV1 transformed cells, choline, phosphocholine, spermine and glutathione, among others, were down-regulated, whereas creatine, dopamine, arginine and AMP were present at higher levels. Using similar approach, Shrestha *et al.* studied the metabolic effect induced by Kaposi's sarcoma-associated herpesvirus (KSHV) in latently infected B-lymphocytes (BCBL-1).[120] Both BCBL-1 and reference non-KSHV infected B-lymphocytes (BJAB) were grown in suspension under the same conditions. Mass spectra of BCBL-1 and BJAB cells were found different with respect to the relative signal intensity of several signals, mostly corresponding to phospholipids (Fig. 6a). Thus, several phosphatidylcholines were downregulated in virally-infected cells (Fig. 6b). The largest difference in expression between the two cell types was observed for a low molecular weight protein with a nominal monoisotopic mass of 4960 Da, which was tentatively assigned as thymosin b4 (Tb4). Expression of Tb4 was reduced by greater than 90% in BCBL-1 cells with respect to BJAB cells. These studies point to metabolic pathways that have a heretofore unexplored role in the viral transformation of host cells and demonstrate that, after the observed biomarkers are validated with functional studies, LAESI-MS could serve as a rapid screening platform for early diagnosis of disease in humans.

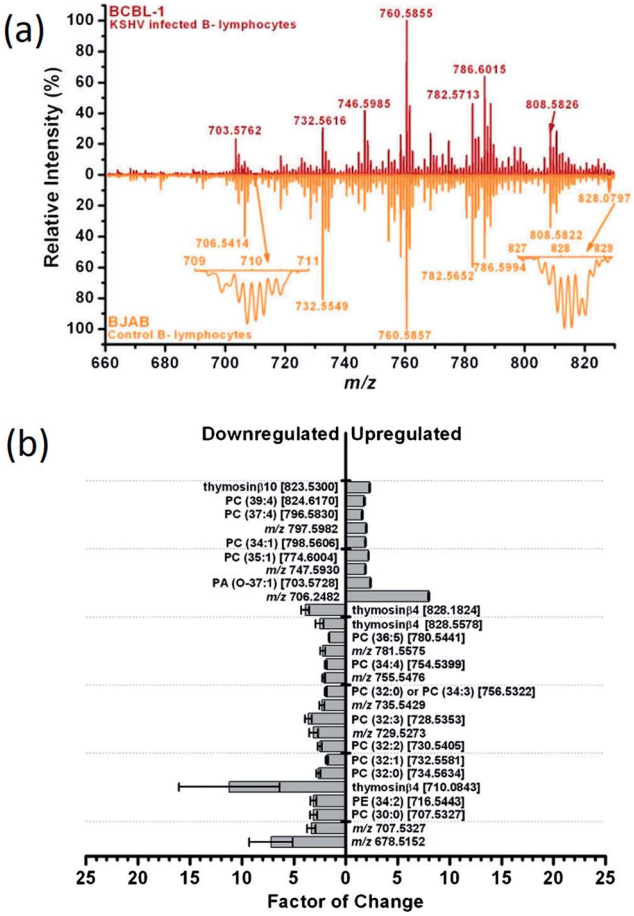

Fig. 6 (a): Positive ion mode LAESI mass spectra pertaining to the lipid region (*m/z* 660–830) in KSHV infected B-lymphocytes BCBL-1 (top) and non-infected B-lymphocytes BJAB (bottom). (b): Identification of ions having the highest contribution to the spectral differences. The grey bars represent the relative up- and down-regulation of compounds in BCBL-1 cells with respect to BJAB cells. Indicated on each bar is the standard error of the mean value, the name of the identified compound, and its *m/z* value. Adapted from ref. 120.

Emerging trend: single-cell analysis

Single-cell analysis is of great importance in mechanistic and clinical studies, because individual cells within a colony or tissue often have different chemistry and functions. MS is a popular approach for single-cell analyses owing to the high sensitivity, high chemical specificity and broad molecular range of detection.[134–137] Most common ionization techniques employed in this field include LDI, MALDI, secondary ionization mass spectrometry (SIMS), and inductively coupled plasma (ICP).

Recent studies show that the single-cell MS analysis can be performed with substantial depth of chemical information using ambient sampling. This became possible owing to the steadily improving sensitivity of modern mass spectrometers and increasing spatial resolution of ionization techniques. Compared to vacuum-based methods, AMS allows faster and easier profiling of individual cells on a surface.

Using LAESI-MS with ~30 μm ablation spot, Shrestha *et al.* explored cell-to-cell variations in epidermal cells of an *Allium cepa* onion bulb and a *C. aurantium* leaf, as well as in human buccal epithelial cells.[127] Based on the ion intensity profiles for particular chemicals, it was concluded that the microablation of a cell using a sharpened optical fiber did not notably affect the metabolite composition of the adjacent cells. Thus, secondary metabolites associated with pigmentation, such as cyanidin and quercetin, were found to be specifically localized in the pigmented epidermal cells. In contrast, sucrose was distributed uniformly throughout all the studied cells with slightly higher intensities in the nonpigmented cells. Interestingly, alliin, which is a precursor metabolite responsible for the smell of onion, was concentrated in only 2 cells and absent in the other cells. In a follow-up study from the same group, Stolee *et al.* profiled the distribution of metabolites at the subcellular level using a combination of microdissection and *in situ* LAESI–MS detection.[126] Large metabolite gradients were revealed between the cytoplasm and nucleus of *A. cepa* epidermal cells.

Using DESI-MS, Ferreira *et al.* found lower variation of phospholipids in individual unfertilized mouse oocytes compared to blastocysts.[138] The increased heterogeneity of lipid profile during mouse preimplantation development can reflect functional and structural specialization by the blastocyst membrane. DESI-MS of embryos cultured *in vitro* displayed more homogeneous lipid profile compared to the embryos grown *in vivo* owing to the nutrient restriction in the culture medium. In a follow-up study, González-Serrano *et al.* used DESI-MS to profile individual bovine oocytes and blastocysts for free fatty acids, phospholipids, cholesterol-related molecules, and triacylglycerols.[139] Statistical analysis on DESI-MS data allowed unequivocal discrimination between oocytes and blastocysts based on specific lipid profiles. DESI-MS combined with transcript regulation analysis and transcript regulation revealed significant difference in homeostasis of cholesterol and FFA metabolism for *in vitro* and *in vivo* grown blastocysts.

Date *et al.* utilized a nanoelectrospray tip as a pipet to extract the contents of subcellular regions. The sampled cell sap was directly nebulized/ionized by applying high voltage to the capillary tip at the entrance of a mass spectrometer.[140] This allowed the metabolic study of anti-breast cancer drug, tamoxifen, in a single human hepatocellular carcinoma cell, HepG2, at the subcellular level. Nonmetabolized tamoxifen was present in both the cytoplasm and a vacuole, while its metabolites were only found in the cytoplasm. A number of single-cell analyses relying on similar approach were reported recently.[140–142] For example, Gholipour *et al.* used a sharp capillary to sample and ionize chemicals in parenchyma cells of tulip bulb.[143] Single-cell metabolite profile for the bulbs of *Tulipa gesneriana* stored at 25 °C and at 15 °C was examined. Several sugars, amino acids, organic acids, vitamins, fatty acids, and secondary metabolites were identified.

At present, the major limitations of single-cell AMS analysis are related to the sensitivity of mass spectrometers and spatial resolution of sampling. The lower sensitivity of AMS detection compared to vacuum methods generally limits the single-cell studies to large plant cells (>30 μm). Smaller vertebral cells contain lower number of molecules and therefore require higher sensitivity of detection. Besides that, the analysis of small individual cells (~10 μm) in a tissue or colony is difficult to achieve due to the larger size of ablation spot in most AMS techniques. To ensure the single-cell mode of sampling, interrogated cells should be relatively distant from each other. This complicates localization of single cells during the analysis. Finally, the limited lateral resolution of ambient techniques generally prevents detailed single cell imaging, although certain chemical information on subcellular level can be obtained.[140]

Concluding remarks

With the use of ambient sampling, MS analysis of *in vitro* grown cells can be performed with much higher speed, throughput and operational ease compared to the traditional methods. A variety of approaches have been developed over recent years that allow direct and noninvasive chemical characterization of microbial cultures and mammalian cell lines without any preparatory steps required. Recent studies strongly indicate the potential of AMS for the molecular fingerprinting of cells and biomarker discovery. Noninvasive sampling in real time enables visualization of cell adaptation to external factors such as drug/antibiotic treatment or changes in the culturing conditions. Finally, VOC biomarkers of pathogens and cancer cells identified *in vitro* can be directly referenced to the *in vivo* chemical fingerprints of expired breath or skin vapors from animals and humans, thus providing a tool for specific and noninvasive clinical diagnostics. The past 2–3 years were the most fruitful for the AMS research on cell cultures, so the current progress is likely to hold in near future.

Acknowledgements

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