High-density micro-arrays for mass spectrometry†

Pawel L. Urban,[a] Konstantins Jefimovs,[b] Andrea Amantonico,[a] Stephan R. Fagerer,[a] Thomas Schmid,[a] Stefanie Mädler,[a] Josep Puigmarti-Luis,[a] Nils Goedecke[c] and Renato Zenobi*[a]

Received 19th July 2010, Accepted 21st September 2010
DOI: 10.1039/c0lc00211a

Functional high-density micro-arrays for mass spectrometry enable rapid picolitre-volume aliquoting and ultrasensitive analysis of microscale samples, for example, single cells.

High-throughput analysis of the molecular composition of cells and low-volume samples is important in a variety of fields. It can be achieved using micro-array technology in conjunction with fluorescence readout.[1,2] However, the requirement for fluorescent tags puts a limit on the type and the number of molecules that can be analyzed. Parallel detection of chemically diverse species requires a more powerful detection technique, such as mass spectrometry (MS).[3,4] Here we describe functional micro-arrays which enable high-throughput analysis of unlabeled molecules in single cells and picolitre-volume samples by mass spectrometry.

High-density micro-arrays for mass spectrometry (MAMS) are fabricated by picosecond-laser ablation patterning of a hydrophobic or "omniphobic" layer coated on a conductive support, such as steel or indium tin oxide (Fig. 1). MAMS are subsequently applied to the analysis of (i) metabolites in single cells and (ii) compounds of biological interest (bioactive peptides, a drug and an intact protein) in picolitre-volume aliquots.

The high-density microscale pattern on the MAMS surface enables unsupervised aliquoting of very small volumes of solutions or suspensions (Fig. 1). This sample preparation strategy completely eliminates the need for using microscale dispensing tools, such as microspotters, to digitize samples prior to MS analysis. It can be very useful, for example, in high-throughput analysis of individual cells by matrix-assisted laser desorption/ionization (MALDI)-MS.

Single-cell mass spectrometry with micro-arrays

Analysis of metabolites in single cells can provide information relevant for biochemical studies that could never be gathered by analyzing bulk samples consisting of many cells.[5,6] Even genetically identical cells can exhibit different phenotypes.[7–9] In order to study phenomena related to cell individuality and cell population heterogeneity, molecular systems biology requires an analytical platform that enables non-targeted molecular analysis of single cells.[10–13]

As shown in Fig. 2, using MAMS, several metabolites could readily be measured in single cells of various unicellular organisms. With the ionization conditions chosen (MALDI matrix: 9-aminoacridine and negative ion mode), the mass spectral peaks corresponding to several metabolites pertaining to the central metabolic pathway are most prominent. Peak amplitudes scale with the number of cells present in different recipient sites (Fig. 2B and C). With this approach, it was possible to conduct mass spectrometric analysis of single cells of a relatively small unicellular organism, baker's yeast (*Saccharomyces cerevisiae*), Fig. 2C. This should provide a basis for integration of existing (*e.g.* genomic and proteomic) data on this model organism with metabolomic data obtained by MS. The sensitivity is such that it will no longer be necessary to average MS signals from hundreds or thousands of metabolically dissimilar cells.

While fluorescent probes expressed in cells enable detection of a limited number of pre-selected small molecules in single cells,[14,15] MS does not restrict single-cell analysis to pre-defined target analytes,[10,12] which is evident when applying MAMS to cells of various biological species (Fig. 2 and 3A). Using MAMS, it was possible to detect adenosine triphosphate (ATP), uridine diphosphate glucose (UDP-Glc)—simultaneously with an abundant phospholipid

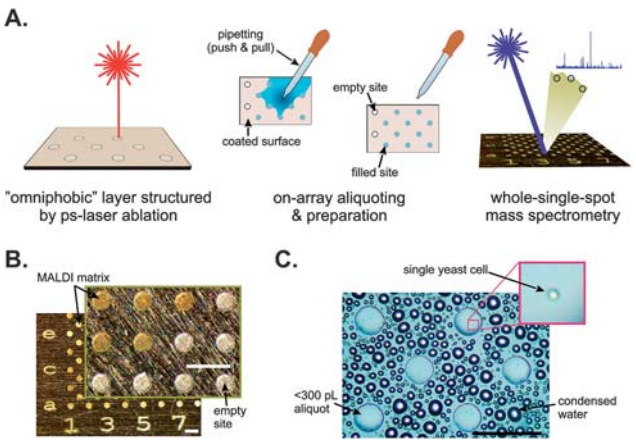

Fig. 1 Micro-arrays for mass spectrometry: (A) general workflow for fabrication and application of MAMS. (B) Homogeneous crystallization of a MALDI matrix (9-aminoacridine) aliquoted on a non-transparent MAMS with a dense arrangement of recipient sites (inset rotated by 45°). The matrix solution is applied with a standard micropipette ("push and pull" method). (C) A transparent MAMS (based on indium tin oxide glass) useful for analysis of small cells and particles. Yeast cells (<10 μm) distributed among ~100 μm recipients; a high ambient humidity (generated with an ultrasonic humidifier). Single cells are present in some of the recipients filled with culture medium (*cf.* inset), while no cells are present on the adjacent coated area. Water condensation can be seen between the recipient sites. Scale bars: 300 μm.

[a]*Department of Chemistry and Applied Biosciences, ETH Zurich, CH-8093 Zurich, Switzerland. E-mail: zenobi@org.chem.ethz.ch; Fax: +41 44 6321292; Tel: +41 44 6324377*
[b]*Laboratory for Electronics/Metrology/Reliability, EMPA, Swiss Federal Laboratories for Material Science and Technology, CH-8600 Dübendorf, Switzerland*
[c]*Department of Biosystems Science and Engineering, ETH Zurich, CH-4058 Basel, Switzerland*

† Electronic supplementary information (ESI) available: Experimental details, additional results and discussion, and additional figures. See DOI: 10.1039/c0lc00211a

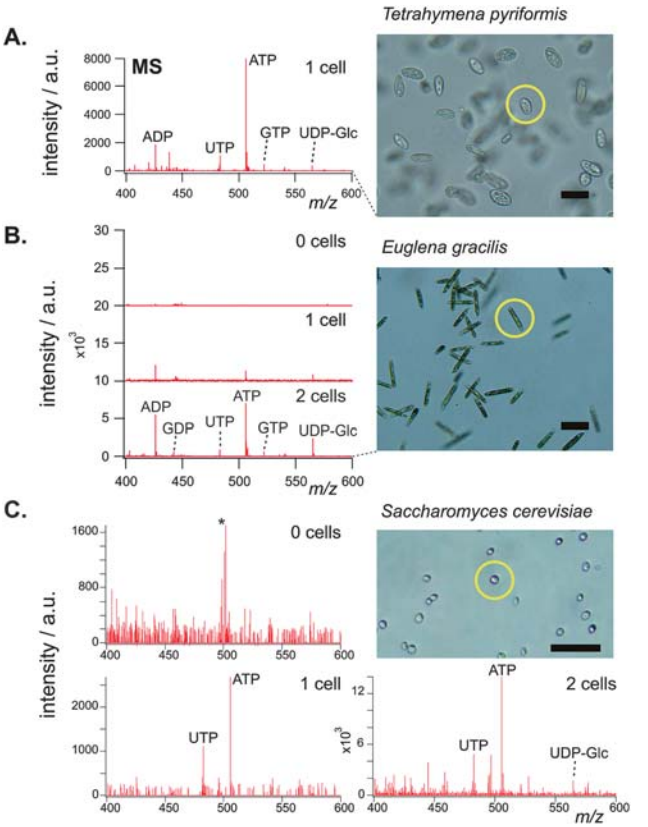

Fig. 2 Application of MAMS to single-cell analysis; MALDI-MS spectra of metabolites from various types of cells with different average size: (A) *Tetrahymena pyriformis*, (B) *Euglena gracilis*, and (C) *Saccharomyces cerevisiae*. Analysis using opaque (A and B) or transparent (based on indium tin oxide glass, C) MAMS. The feature marked with an asterisk (*) in (C) corresponds to the matrix background. Scale bars: 50 μm.

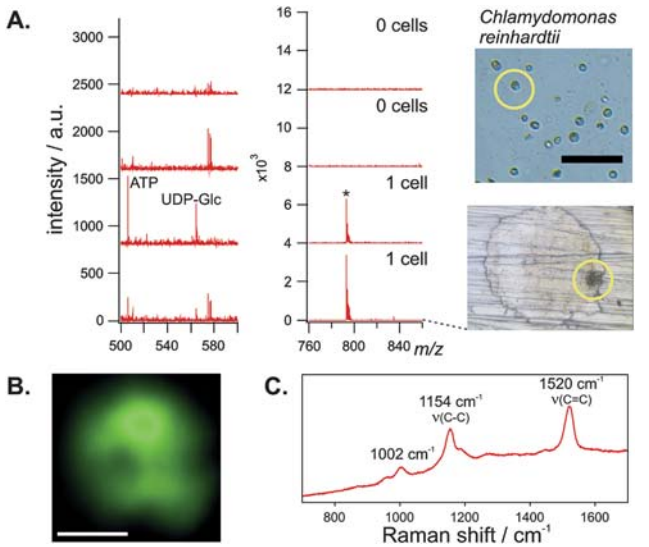

Fig. 3 Analysis of single cells of *Chlamydomonas reinhardtii* by MS and complementary techniques, aided by non-transparent 50 μm MAMS. The peak marked with an asterisk (*) in (A) is assigned to phosphatidylglycerol. The lower microphotograph in (A) shows an optical image of a single *C. reinhardtii* cell on MAMS prior to MALDI-MS analysis (represented in the bottom mass spectrum in (A)) and (B) the corresponding chlorophyll fluorescence image ($\lambda(em)_{max}$ = 684 nm). (C) Raman spectrum of a single *C. reinhardtii* cell on the MAMS with the bands characteristic of β-carotene. Scale bars: (A) 50 and (B) 5 μm.

species—in single cells of unicellular algae, *Chlamydomonas reinhardtii* (Fig. 3A). Although the intracellular concentration of ATP is relatively high (usually, low mM range), it is the small volume of cytoplasm (femtolitres) that makes detection of the analyte extracted from relatively small cells (attomoles) difficult. Here, obtaining mass spectra from such microscale samples (Fig. 2C and 3A) was feasible since the minute amount of a cell's metabolome was not dispersed over an area considerably larger than that of the laser beam focus used for ionization.

In this work, application of the MALDI matrix solution onto the cells distributed on MAMS was facilitated with an ultrasonic spray (see ESI† for details); the microdroplets, landing on the MAMS close to the recipients, merged with the bigger droplets (adhering to the wettable area), leaving the coated area clear. Considering the variety of MALDI matrices available, we believe that this general approach can be extended to other classes of sufficiently abundant compounds present in cells and may provide a useful tool for future studies on single-cell metabolomics.

Using MAMS, one can easily combine MS with other standard detection techniques, *e.g.*, fluorescence (Fig. 3B) and Raman spectroscopy (Fig. 3C). To demonstrate this, the presence of two natural cell components (chlorophyll and β-carotene) was verified in a single cell deposited on the MAMS. By simply changing the laser wavelength, it should be possible to evaluate levels of expression of green fluorescent protein and relate them to the MS signals of other compounds present in the same cell. Such a synergy of different detection techniques makes MAMS ideal for studies in which multiple features of a cell's phenotype, *e.g.*, its position in the cell cycle and metabolite levels, need to be inter-related.

Handling large numbers of individual cells before and during MS analysis has always been challenging. Although rapid arraying of cells on flat supports can be achieved with various methods (*e.g.* ref. 16–20), to our knowledge, none of these approaches have so far been implemented to study the metabolic composition of single cells by MS. Due to the properties of MAMS (capture of picolitre-volume aliquots of liquid within the high-density pattern), it was neither required to address recipient sites individually, as is the case when using picolitre-volume microdispensers, nor necessary to implement microfluidic channels for distribution of cell suspensions. Deposition on MAMS could be achieved quickly and without exerting much mechanical (shear) stress on the cells, a factor that might need to be considered when implementing some of the other strategies available for cell handling. Dilution of the cell suspensions as well as the speed of the deposition were selected to have a high percentage of recipients filled with single cells. Cells stay alive following the deposition step if evaporation of the picolitre aliquots of the culture medium during cell counting (under a microscope) is prevented by maintaining the MAMS in a humid atmosphere, in which condensation of water occurs (Fig. 1C). The procedure allows one to obtain spectra corresponding to multiple individual cells at a given time point—a prerequisite for studies on cell populations.

While single-cell analysis protocols with optical detection often incorporate microfluidic chips,[10–13,21] the MAMS concept is an inexpensive and practical complement to the microfluidic toolkit, offering easy mass spectrometric readout.

Picolitre-volume mass spectrometry

As shown in Fig. 4, MAMS can be used in conjunction with several MALDI matrices, suitable for analysis of the molecules of interest. Various compound classes (metabolites, peptides, a drug and a large protein) could readily be analyzed with limits of detection in the low attomole range (Fig. 4). Detection limits for three metabolites (Fig. 4A) were as low as ~500 zeptomoles, which should warrant detection of sub-millimolar concentrations of these compounds present in the average-sized yeast cells (<10 μm), even considering possible ion suppression. Besides, ~50 attomoles of an intact protein (bovine serum albumin) as well as its dimer could be detected (Fig. 4D).

The total volume of the sample pipetted onto the MAMS can be very low (down to a few nanolitres) and any excess can simply be pulled away (Fig. 1A). Thus, only picolitre amounts of the aliquoted sample are retained within the individual recipients (Fig. 1C), and all the rest can be recycled for further experiments. This makes MAMS attractive for the analysis of precious samples by MS. The intrinsic picolitre-volume aliquoting decreases the requirements of MALDI-MS with respect to the sample volume used in single measurement, by up to 3 orders of magnitude. MAMS digitize liquid samples, creating multiple aliquots from one sample using a single pipetting step: this partly addresses the concerns related to chemical heterogeneity of MALDI spots prepared using conventional supports and methods.

In addition to the efficient sampling prior to MS, MAMS also implement a known strategy for detecting small amounts of analytes by MALDI-MS, which is based on minimizing the sample presentation area.[22–25] Confinement of every micro-aliquot, down to the dimensions of the MALDI laser beam focus, makes it easy to probe the whole area of the individual regularly structured deposits of the digitized samples (Fig. 1B), without "blind" rastering and searching for "sweet spots" while accumulating noisy subspectra.

Similarly to the analysis of single cells by MAMS, being able to irradiate the entire micrometre-scale sample deposit with the MALDI laser beam offers a considerable advantage to ultratrace MS analysis,[25] since no sample is wasted. In other words, the entire amount of the analyte present in each recipient site may be desorbed within a short period of time. All these allow one to maintain high sensitivity and reliability without sacrificing the analytical throughput.

In this study, MAMS were tested with two commercial MALDI-MS instruments, achieving an acquisition speed greater than 2 samples per second. The compatibility with the standard MALDI-MS technique is also due to the fact that MAMS are electrically conductive, which prevents MS targets from unfavorable electrostatic charging. The surface density of the 100 μm MAMS is ~1250 sample recipients per cm^2, which provides a very high utilization of the sample support area (~250× greater than in commercial MALDI plates).

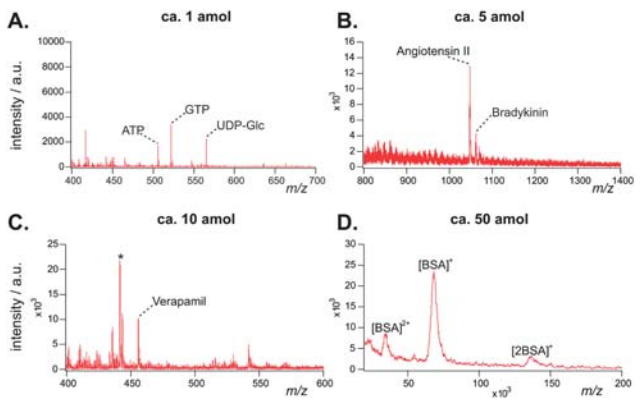

Fig. 4 Low-attomole-level sensitivity observed for a variety of analytes using the 50 μm MAMS: (A) three primary metabolites (ATP, GTP and UDP-Glc, negative ion mode, and matrix: 9-aminoacridine), (B) two peptides (angiotensin II and bradykinin, positive ion mode, and matrix: α-cyano-4-hydroxycinnamic acid (CHCA)), (C) verapamil (positive ion mode and matrix: CHCA), and (D) bovine serum albumin (BSA, positive ion mode with high-mass detector, exponential smoothing, and matrix: sinapinic acid). MALDI-MS instrument: AB Sciex 4800. The feature marked with an asterisk (*) in (C) corresponds to the matrix background.

Outlook

We implemented the high-density micro-arrays with MALDI-MS, but it is also imaginable to use MAMS in conjunction with matrix-free ionization methods, such as the nanostructure-initiator mass spectrometry,[26] as well as the recently introduced laserspray ionization.[27] In the future, the versatility of MAMS can facilitate high-throughput proteomic analysis and screening of biomolecular complexes with minute amounts of samples.

We believe that the high-density MAMS will also find other applications than those described in this article, for example, for collection of effluents from microscale capillaries/microcolumns or microfluidic devices for MS, analysis of compounds adsorbed on single chromatographic beads, analysis of cancer and stem cells, high-throughput combinatorial screening, or calibration of surface analysis techniques. The ability to maintain cells alive within droplets trapped in separate recipients of the MAMS for a certain period of time may also enable studies on the chemical communication within microbial cell populations, for example, the so-called "quorum sensing".

Acknowledgements

We thank K. Barylyuk, C. Bärtschi, H. Benz, L. Bertschi, E. Beurer, H. K. Bui, R. Häfliger, F. Krumeich and J. Stadler for their assistance. This research was supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (PIEF-GA-2008-219222—MESEL).

References

1 *Microarrays: Preparation, Microfluidics, Detection Methods, and Biological Applications*, ed. K. Dill, R. H. Liu and P. Grodzinsky, Springer, New York, 2009.
2 T. G. Fernandes, M. M. Diogo, D. S. Clark, J. S. Dordick and J. M. S. Cabral, *Trends Biotechnol.*, 2009, **27**, 342–349.
3 G. Marko-Varga, S. Ekström, G. Helldin, J. Nilsson and T. Laurell, *Electrophoresis*, 2001, **22**, 3978–3983.
4 A. J. Ibáñez, A. Muck, V. Halim and A. Svatoš, *J. Proteome Res.*, 2007, **6**, 1183–1189.
5 H. M. Davey and D. B. Kell, *Microbiol. Rev.*, 1996, **60**, 641–696.
6 A. Amantonico, P. L. Urban, S. R. Fagerer, R. Balabin and R. Zenobi, *Anal. Chem.*, 2010, **82**, 7394–7400.
7 J. R. S. Newman, S. Ghaemmaghami, J. Ihmels, D. K. Breslow, M. Noble, J. L. DeRisi and J. S. Weissman, *Nature*, 2006, **441**, 840–846.
8 P. J. Choi, L. Cai, K. Frieda and X. S. Xie, *Science*, 2008, **322**, 442–446.
9 Y. Taniguchi, P. J. Choi, G. W. Li, H. Chen, M. Babu, J. Hearn, A. Emili and X. S. Xie, *Science*, 2010, **329**, 533–538.

10 *Single Cell Analysis: Technologies and Applications*, ed. D. Anselmetti, Wiley-VCH, Weinheim, 2009.
11 A. Amantonico, P. L. Urban and R. Zenobi, *Anal. Bioanal. Chem.*, 2010, DOI: 10.1007/s00216-010-3850-1.
12 *Chemical Cytometry: Ultrasensitive Analysis of Single Cells*, ed. C. Lu, Wiley-VCH, Weinheim, 2010.
13 D. G. Spiller, C. D. Wood, D. A. Rand and M. R. H. White, *Nature*, 2010, **465**, 736–745.
14 M. Fehr, W. B. Frommer and S. Lalonde, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 9846–9851.
15 J. Berg, Y. P. Hung and G. Yellen, *Nat. Methods*, 2009, **6**, 161–166.
16 M. Hosokawa, A. Arakaki, M. Takahashi, T. Mori, H. Takeyama and T. Matsunaga, *Anal. Chem.*, 2009, **81**, 5308–5313.
17 Y. K. Kim, S. R. Ryoo, S. J. Kwack and D. H. Min, *Angew. Chem., Int. Ed.*, 2009, **48**, 3507–3511.
18 N. Ferrell, D. Gallego-Perez, N. Higuita-Castro, R. T. Butler, R. K. Reen, K. J. Gooch and D. J. Hansford, *Anal. Chem.*, 2010, **82**, 2380–2386.
19 Y. Gong, A. O. Ogunniyi and J. C. Love, *Lab Chip*, 2010, **10**, 2334–2337.
20 J. Y. Shiu, C. W. Kuo, W. T. Whang and P. Chen, *Lab Chip*, 2010, **10**, 556–558.
21 J. El-Ali, P. K. Sorger and K. F. Jensen, *Nature*, 2006, **442**, 403–411.
22 L. Li, R. E. Golding and R. M. Whittal, *J. Am. Chem. Soc.*, 1996, **118**, 11662–11663.
23 J. Franzen and M. Schürenberg, patent publication no. GB2332273, 1999.
24 M. Schürenberg, C. Luebbert, H. Eickhoff, M. Kalkum, H. Lehrach and E. Nordhoff, *Anal. Chem.*, 2000, **72**, 3436–3442.
25 A. Amantonico, J. Y. Oh, J. Sobek, M. Heinemann and R. Zenobi, *Angew. Chem., Int. Ed.*, 2008, **47**, 5382–5385.
26 T. R. Northen, O. Yanes, M. T. Northen, D. Marrinucci, W. Uritboonthai, J. Apon, S. L. Golledge, A. Nordström and G. Siuzdak, *Nature*, 2007, **449**, 1033–1036.
27 C. N. McEwen, B. S. Larsen and S. Trimpin, *Anal. Chem.*, 2010, **82**, 4998–5001.