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Mass Spectrometry-based characterization of endogenous peptides and metabolites in small volume samples

Ta-Hsuan Ong, Emily G. Tillmaand, Monika Makurath, Stanislav S. Rubakhin, and Jonathan V. Sweedler*

Department of Chemistry and Beckman Institute for Advanced Science and Technology,
University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States

Abstract

Technologies to assay single cells and their extracellular microenvironments are valuable in elucidating biological function, but there are challenges. Sample volumes are low, the physicochemical parameters of the analytes vary widely, and the cellular environment is chemically complex. In addition, the inherent difficulty of isolating individual cells and handling small volume samples complicates many experimental protocols. Here we highlight a number of mass spectrometry (MS)-based measurement approaches for characterizing the chemical content of small volume analytes, with a focus on methods used to detect intracellular and extracellular metabolites and peptides from samples as small as individual cells. MS has become one of the most effective means for analyzing small biological samples due to its high sensitivity, low analyte consumption, compatibility with a wide array of sampling approaches, and ability to detect a large number of analytes with different properties without preselection. Having access to a flexible portfolio of MS-based methods allows quantitative, qualitative, untargeted, targeted, multiplexed, spatially resolved investigations of single cells and their similarly scaled extracellular environments. Combining MS with on-line and off-line sample conditioning tools, such as microfluidic and capillary electrophoresis systems, significantly increases the analytical coverage of the sample's metabolome and peptidome, and improves individual analyte characterization / identification. Small volume assays help to reveal the causes and manifestations of biological and pathological variability, as well as the functional heterogeneity of individual cells within their microenvironments and within cellular populations.

Keywords

Bioanalytical microanalysis; single cell; microenvironments; mass spectrometry

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*Corresponding Author: Jonathan V. Sweedler, Department of Chemistry, 600 S. Mathews Avenue, Urbana, IL 61801, Tel: 217-244-7359, jsweedle@illinois.edu.

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Conflict of interest

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1. Introduction

Advancing our capability to analyze single cells and their microenvironments represents an exciting goal of bioanalytical measurement science. The cell is a central structural and functional unit of most unicellular and multicellular organisms. Methods to assay small volume samples from heterogeneous biological organisms, including the ability to select the most appropriate analytes for a specific investigation while ensuring reduced analyte consumption, can provide important information about cellular function. Depending on the analytical approach used and the specimen being examined, sample sizes may vary widely, from femtoliter to milliliter or larger. Here, we consider samples of less than a microliter as being small volume; these samples can be comprised of fundamental structures such as individual organelles and cells, as well as their extracellular environments.

Why are single cell assays important? One key factor is that the coordinated activities among individual cells contribute to the physiology and behavior of multicellular organisms, and also to the organization of ecological communities in unicellular organisms. Cellular variability, as well as pathological and functional heterogeneities, are integral to the development of individual biological and behavioral traits, and have important roles in the etiology of many diseases. The study of single cells and their microenvironments (i.e., extracellular spaces) aids our understanding of these phenomena, as well as the overall structure, organization and activity of organisms and cellular populations [1–6].

The initial achievements in small sample analysis were associated with the technological progress of microscopy, starting centuries ago with Robert Hooke and Anton van Leeuwenhoek [7]. Visual observations of individual cells led to the development of methods that were used to directly or indirectly determine cellular chemical composition, e.g., the Gram and Golgi cell staining methods. Today, chemical investigations of a diverse range of analyte types can be qualitatively and quantitatively characterized and localized using a plethora of new technologies that employ molecular probes, analyte separation approaches such as chromatography and capillary electrophoresis (CE), microfluidic devices, electrochemical methods, mass spectrometry (MS), and a number of optical and resonance methods [1,8].

When selecting the appropriate tool for a small volume analysis, MS is often the method of choice; it provides low detection limits, multiplexed detection, greater coverage for many analyte classes, and capabilities for nontargeted, targeted, qualitative, and quantitative investigations. Hyphenation of MS to sample conditioning and/or microseparation approaches, such as solid phase microextraction, CE, microfluidics, gas chromatography, and capillary-scale chromatography, can improve the coverage of the peptidome and metabolome and add useful information about the analytes, such as migration time and hydrophobicity.

In this review, we focus on small volume assays of two analyte classes—peptides and metabolites. These compounds influence cellular morphology, energy balance, cell-to-cell communication, and many other cellular functions. There is a caveat to consider when planning a single cell investigation; given the array of molecules and their large dynamic

range, the current limits of detection of most available methods do not allow the characterization of an entire peptidome or metabolome of an individual cell. Nonetheless, one can measure the most abundant and/or detectable analytes present, many of which have important functional roles, e.g., signaling molecules. These include peptides as well as a large variety of small molecules such as lipids, carbohydrates, and amino acids, which often accumulate inside a cell and are released into the surrounding microenvironment under tightly regulated events.

We begin discussion with an overview of the single cell sample preparation process, followed by a section highlighting several commonly used mass spectrometric methods to analyze small volume samples. Next we review the hyphenation of analyte separation techniques to MS, and close with MS-based technologies used to measure cellular microenvironments and releasates.

2. Sample preparation

One issue with single cell measurements is that many typical sample conditioning steps, such as concentration, desalting, and fractionation, can lead to unacceptable sample / analyte loss; thus, choosing the appropriate sampling technique becomes critical to a successful MS investigation. Sampling an individual cell is challenging due to its small scale, chemical complexity, and the structural and chemical instability of the cell and surrounding spaces. Often, as in the case of single neurons and astrocytes, it is difficult to isolate cells such as a neuron or astrocyte in their entirety due to the extensive branching of cellular processes and their intertwining and mechanical interconnectivity with other cells. Additionally, removing a cell from its native environment triggers intracellular biochemical changes. Therefore, special care is required to stabilize the sample by lowering its temperature, adding stabilization agents such as glycerol and trehalose, and decreasing sample collection time. Stringent control experiments are often performed to determine the extent of possible alteration due to sample preparation.

An assortment of MS-compatible sample collection and manipulation approaches are available, including whole cell mechanical dissection, direct cytoplasmic sampling, flow cytometry, optical trapping, and microenvironment collection using a variety of microfluidic devices [9]. Furthermore, some of the approaches, such as optical trapping, can be used to isolate and profile analytes at the subcellular level [10,11]. Among these, microfluidics provides a powerful platform on which all steps of the sample preparation process can be performed, including cell lysis and content conditioning, prior to MS analysis [12]; these devices are also used to collect cell releasate (see Sections 4 and 5 for an expanded discussion of how microfluidics is applied in MS-based investigations).

3. MS-based approaches

A variety of MS-based methodologies have been developed and applied to direct assays of small volume samples: matrix-assisted laser desorption / ionization (MALDI) [13–16], desorption electrospray ionization [17], laser desorption ionization [18], secondary ion MS [19– 22], nanostructure-initiator [23], electrospray ionization (ESI) [24,25], inductively coupled plasma (ICP) [26–29], and laser ablation electrospray ionization [30–32]. Here we

highlight methods that are particularly well suited to such small volume assays: direct single cell profiling using MALDI MS, followed by two high-throughput methods, microarray mass spectrometry (MAMS) and mass cytometry, and MALDI MS imaging (MSI). For more information on these and other technologies for single cell and other small volume investigations, the interested reader is referred to several recent reviews [6,12,33,34].

3.1. Direct MALDI MS

Single cell analysis generates data that can document an individual cell's actual chemical state, rather than a deriving an "average" state based on assay of a cell population. So why aren't single cell measurements performed more routinely? A major challenge is the small sample size and the issues this creates for its chemical characterization. One approach that has been successfully used to assay small volume samples is MALDI MS [14,35,36]. Because of its low detection limits, high salt tolerance and wide molecular mass range of detection, it is one of the most commonly used methods for single cell peptide and metabolite measurements. A MALDI MS investigation generally starts with the identification and removal of the target cell from a population. The isolated cell is exposed to a MALDI matrix solution and the combined sample dried onto a sample plate. Next, the sample is irradiated with a laser to generate a plume of neutral particles and ions, which are subsequently analyzed by a mass analyzer. As each laser shot only consumes a small portion of the sample, one can average the signal from hundreds to thousands of laser shots. Moreover, multiple experiments can be conducted on just one cell. MALDI MS has been used as a complementary technique to confirm known peptide localization, and as a tool for new molecular discovery. Many classes of analytes from a variety of organisms have been assayed with MALDI MS, including DNA, RNA, proteins, peptides, and metabolites.

Several early MALDI MS-based investigations profiled isolated neurons from the central nervous system of the pond snail *Lymnaea stagnalis* [37–39] and isolated dense core vesicles from the sea hare *Aplysia californica* [40]. Molluscan neurons are well suited for single cell analysis and method development because they are relatively large and are involved in welldefined physiological functions. These cells are variable in size, with cell body diameters ranging from 10–1000 μm . The larger cells are appropriate targets for individual isolation and manipulation; not only do they contain easier to measure volumes, but they are similar to smaller cells in terms of their mechanisms of cell functioning. As the difference in analyte amounts from the smaller to larger cells is a million fold, this increased volume enables many measurements. Beyond mollusks, there are many examples of peptide-content studies using smaller cells. For example, individual rat pituitary cells were isolated and profiled for the presence of cell-to-cell signaling peptides [8,41]. Peptides from the pro-opiomelanocortin (POMC) and cocaine-and-amphetamine-regulated transcript prohormones were detected, consistent with the known peptide content of the pituitary.

While typically used to identify compounds, MALDI MS can also generate quantitative information from an individual cell. Several quantitative single cell MALDI MS approaches were described using *A. californica* neurons [42]. In these approaches stable isotope analyte labeling allowed measurement of relative peptide content, and standard addition was

conducted for absolute quantitation. Quantitative analysis of small volume samples with standard addition is possible because MALDI MS consumes only a fraction of the sample per measurement, allowing sequential data collection after each addition of standard and sample reconstitution.

Another interesting application of MALDI MS involves using tandem MS (MS/MS) for the structural characterization of peptides, which can be challenging because typically MS/MS requires larger analyte amounts than direct MS profiling. In one notable example, Bai and colleagues [43] identified several D-amino acid-containing peptides in abdominal ganglia neurons from *A. californica*. These peptides exhibit the unusual post-translational modification in which one amino acid residue is isomerized from the L-form to the D-form. While isomerization does not introduce a mass shift, the reported results showed that the MS/MS spectra contained differences in the intensity of the fragment ions sufficient for the identification and quantification of the studied D-amino acid-containing peptides.

Several MALDI MS-based studies have incorporated other analytical approaches to confirm analyte identification and localization. Jarecki et al. [44] demonstrated the efficacy of single cell MALDI MS for the detection of low abundance peptides in rare cells. They compared peptides detected in ALA and RID neurons from the dorsal ganglion of the nematode *Ascaris suum* with known peptides previously found using immunocytochemical and *in situ* hybridization approaches; the results from all three methods were in good agreement. Six novel peptides were also observed and sequenced via MS. The complementary nature of different analytical approaches is further illustrated in two studies on insect neurons [15,45]. The neuropeptidome of the antennal lobe of the American cockroach *Periplaneta americana* was investigated with MALDI MS by systematically reducing the sample size from whole tissue to single cells [15]. Dissected tissues were first profiled via MALDI MS to get an overview of their neuropeptide content. Single glomeruli, cell clusters, and neurons were then progressively interrogated in combination with immunolabeling to obtain data on the peptide profiles of different regions of the specimen. However, immunolabeling typically requires a fixation step that negatively affects the MS detection of many compounds. To resolve this issue, Neupert et al. [15] created a workflow to facilitate MALDI MS analysis after immunostaining. By adding a mixture containing 2,4-dinitrophenylhydrazine and α -cyano-4-hydroxycinnamic acid and heating the sample, the crosslinks formed by fixation can be destroyed, freeing analytes for MS detection. Together these studies show that immunocytochemical staining is useful for localizing relevant cells for MALDI MS analysis [15,44,45]. Advances like this broaden the applicability of MALDI MS single cell profiling to more biological systems.

3.2. MALDI MSI

MALDI MSI is an information-rich technique that adds spatial information to MS data sets. It is commonly performed in the microprobe mode in which the laser beam is rastered across the sample to collect position-resolved mass spectrometric information [46–48]. Although the spatial resolution of MALDI MSI is often limited by laser dimensions (typically 15–75 μm), and by having a sufficient amount of analyte to detect within the laser spot, the investigation of cells at subcellular scales is becoming possible. Currently, MALDI MSI

spatial resolution pales in comparison with the resolution attained by optical microscopy; however, the molecular information acquired makes single cell MALDI MSI an important approach to have in one's analytical armamentarium.

There are many examples showing the capability of MALDI MSI to reveal the subcellular localization of analytes and cell-to-cell chemical heterogeneity within a tissue section. One early study used single cell MALDI MSI to investigate a culture of *A. californica* bag cell neurons [49]. Even at 50 μm lateral resolution, the cell body and neuronal processes could be resolved based on peptide signal. More recently, MSI experiments have been performed at <10 μm spatial resolution using a laboratory-built instrument [50–53]. Mouse pituitary sections were imaged at 5 μm resolution [51] and a distribution of the POMC peptide, α -melanocyte-stimulating hormone, between two mixed cell types within the intermediate pituitary was observed, illustrating how single cell multiplexed peptide information can be obtained without cell isolation from tissue. In another study, HeLa cells were imaged at 7 μm spatial resolution, demonstrating that an instrument with high mass resolution and high mass accuracy can improve molecular image contrast and specificity by reducing interference from compounds with similar m/z ratios [52]. High spatial resolution using a custom built mass spectrometer was also achieved by Zavalin et al. [54]. They imaged the distribution of lipids in human embryonic kidney 293 (HEK-293) and RKO cells at nearly 1 μm spatial resolution. They also showed the potential for subcellular peptide and protein MSI in a profiling experiment that showed insulin localization to the cytoplasm of human pancreas islet cells.

It is possible to obtain a higher spatial resolution than the diameter of the laser beam footprint. In one example, a network of *A. californica* neurons was cultured on a stretchable substrate [55]. Prior to MSI, the network was broken apart into individual pieces containing single cells by stretching the substrate. Spatially targeted acquisition of mass spectra and implementation of a molecular image reconstruction step allowed for the determination of the original distribution of analytes in the network of single cells. Both MS and MS/MS experiments were performed using the approach, thus aiding neuropeptide identification.

Oversampling is another method that improves spatial resolution [56]. By depleting the sample with the laser at the starting point and stepping the laser by less than the beam diameter, analyte signal will only come from the sample region that had not previously been exposed to laser irradiation, thus improving spatial resolution. Jurchen et al. [56] developed this method using individual neurons deposited on an electron microscopy calibration grid and traditional UV laser microprobe MALDI MSI. Features of the grid cannot be resolved when the raster step is 100 μm and laser beam footprint is $\sim 100 \times 200 \mu\text{m}$. However, both the grid's wires (40 μm wide) and the holes (60 μm wide) become visible when the laser is rastered at 25 μm and oversampling is used. A $\sim 100 \mu\text{m}$ neuron from *A. californica* was imaged using this approach. Changes in peptide signal intensity between adjacent pixels was observed, demonstrating that distribution of analytes at a spatial resolution smaller than the laser beam diameter can be imaged.

While impressive advances have been made, single cell laser microprobe MSI tends to have a low throughput because hundreds or even thousands of locations have to be examined one

at a time. Stigmatic imaging presents an alternative that can bypass some of these problems. The sample is irradiated with a diffused laser and specialized ion optics preserve the generated ions' relative two-dimensional spatial localization before their detection by a position-sensitive detector. The Heeren group has developed—and is continually improving—mass microscopes that allow this detection modality [57–61]. Several types of samples have been imaged using the mass microscopes [58,62–64]. In one study, pituitary gland sections from rats, mice, and humans were imaged with 4 μm resolving power [62]; known pituitary peptides were detected and localized for all three sample types. Stigmatic imaging also can be done with infrared lasers, permitting the use of water that is endogenous to the sample as a substitute for classical MALDI matrixes [64]; the boundary between the liver and the stomach of a bait fish was also investigated with 10 μm lateral resolution using this approach. The tradeoff with mass microscopy is that ions in a relatively narrow m/z range are detected in each image so that the chemical information is reduced although the spatial resolution and imaging speed are increased.

3.3. MAMS

The typical single cell MS experiment reveals important information on the chemical composition of a few cells within a large population. However, how representative these cells are for the population is not always known. Even in a supposedly homogeneous population, individual cells differ from each other based on a variety of factors, e.g., different stages of the cell cycle. Many biological phenomena, such as drug resistance or malignant cell transformations, may begin with rare events that can be missed when only a small number of cells in a population are interrogated. A thorough understanding of a biological system's functioning requires high-throughput single cell analysis, which can generate population level data from individual cells. In fact, the cellular-scale MSI methods discussed in the previous section can generate this type of output because hundreds to thousands of cells can be analyzed in one experiment. However, intact tissues or their sections are difficult targets, in part, because it is challenging to align each cell with the laser microprobe automatically. As a result many of the acquired mass spectra will contain signals from multiple neighboring cells and extracellular regions. Analyte migration between cells during MALDI matrix application can also be a confounding factor. Therefore, methods that attain spatial separation between many individual cells and offer highly parallel sample preparation prior to MALDI MS analysis have been developed.

One such approach is MAMS, which is conducted on an array of hydrophilic wells surrounded by an omniphobic material [65]. With the proper cell suspension density, it is possible to deposit single cells into wells by pipetting the suspension onto the array, allowing them to sediment, and removing excess liquid. As result, many wells can be quickly and repeatedly loaded for high throughput and quantitative experiments [66]. The number of cells deposited in a well follows a Poisson distribution and can be visualized using a microscope [67]. Once optimized, each well should contain only a few cells, with a subset containing none or individual cells.

Using the MAMS approach, the metabolite profiles of a population of the yeast *Saccharomyces cerevisiae* were investigated [67]. Visual inspection showed that the MAMS

wells contained 1–15 cells, with ~50% containing three cells or fewer. Two subpopulations with different levels of fructose-1,6-biphosphate were found, reflecting differences in glycolytic fluxes in the cells of the subpopulations.

MAMS investigation of cellular populations can be enhanced by additional bioanalytical examination of the same cells using Raman and fluorescence measurements. By employing these multi-method approaches, metabolomic characteristics of the transition of the algal cells *Haematococcus pluvialis* from the motile to the dormant state were revealed [68]. The dormant stage of *H. pluvialis* was characterized by an accumulation of the red pigment astaxanthin and a decrease of the adenosine triphosphate to adenosine diphosphate ratio, indicating a lowering of the energetic state of dormant cells. A metabolically unique cell was also detected, illustrating the capability to find rare cells with high throughput methods.

3.4. Mass cytometry

Another high throughput method is mass cytometry, which combines flow cytometry with a specially designed ICP-MS system [26,27,69]. This approach is distinct from those discussed so far in two ways; first, it presorts the cells using flow cytometry, and second, the mass spectrometer detects immuno-labeled analytes that contain differentiable labels. The approach is similar to fluorescent flow cytometry and requires one to preselect the analytes of interest before the experiment. While flow cytometry with fluorescent detection is capable of analyzing thousands of cells per minute, the spectral overlap of common fluorescence probes restricts the number of separate antibodies that can be used. Mass cytometry addresses this problem by using antibodies labeled with distinct stable isotopes. Thirty or more antibodies labeled with different isotopes can be used simultaneously to detect this number of analytes, including peptides, proteins, and some metabolites in a single cell, providing more analytical channels than fluorescence-based flow cytometry. This technique allows for the design and implementation of extensive barcoding schemes to enable the assay of a range of analytes, practically limited by the availability of specific antibodies and analyte concentration. Thousands of labeled cells from cell populations can be analyzed in one experiment. Importantly, the approach can determine absolute quantities of the stable isotope labels to provide high throughput quantitative data.

Mass cytometry has been applied to a variety of biological and analytical sciences [26,70–76]. One of the most extensive applications has been to study immune cells. For example, lymphocytes, the cells of the immune system, possess a wide range of antigen specificities [76]. Recently, Bendall et al. [26] analyzed the immune cell population in bone marrow during hematopoiesis. A total of 24 distinct cellular populations were detected using 13 surface expression markers. Additionally, differences in responses to stimuli between these cell types were investigated using 18 intracellular markers. This study was later expanded to include measurements of cell cycle-related parameters [70].

Not surprisingly, specific major immune cell types can be also distinguished and characterized using mass cytometry. Over 200 functional phenotypes of CD8⁺ T cells were identified by measuring 28 parameters representing surface markers and cytokines, among others [75]. Horowitz et al. [71] analyzed natural killer cells using 37 parameters. These results indicated the presence of between 6,000 to 30,000 cellular phenotypes within an

individual. These studies also show that with high throughput, multiplexed analytical approaches, single cell analysis can generate deep insight into the organization of the cellular subpopulations of complex biological systems.

4. Analyte separation techniques hyphenated to MS

The chemical complexity of single cells makes comprehensive characterization of their peptide and metabolite content in one MS measurement challenging. The competitive nature of ionization, limited dynamic range of MS instruments, and relatively low mass resolution of the most sensitive mass spectrometers, restrict the number of analytes that can be directly analyzed in a sample. To improve analyte coverage and identification, a variety of separation and preconcentration methods can be hyphenated to MS detection. CE, capillary liquid chromatography, and microfluidics are frequently incorporated into the sample separation and conditioning process. Providing the capability to analyze small volumes while minimizing analyte loss are key features of these approaches. Some methods that include injection of the entire cell lysate or the extracellular environment into a capillary or a microfluidic device increase the ability to collect a greater fraction of the analyte content. The comparable internal volumes of the analytical devices and the analyzed cells or cellular environments result in minimal sample dilution [9,77–79].

Capillary zone electrophoresis is a useful approach for analyte separation and introduction of the sample into the mass spectrometer via nanoESI ion source [80]. Many endogenous peptides and metabolites exhibit different electrophoretic mobilities in MS-compatible CE buffers such as 1% formic acid. CE not only separates analytes but also provides information on their migration times, which in combination with fragmentation patterns obtained using MS/MS, allows endogenous analyte identification with high levels of confidence. Analyte fragmentation in MS/MS is achieved using a variety of techniques such as collision-induced dissociation, electron capture dissociation, electron transfer dissociation, and postsource decay. These techniques are often used to obtain key structural information on detected metabolites and peptides, including post-translational modifications [5], and can be applied in small volume analysis. CE-MS and CE-MS/MS demonstrated good performance in the investigation of a single cell metabolome [81], with more than 300 molecular features detected in a single neuron and 30 metabolite identities confirmed. The approach allows different types of neurons and their functional conditions/states to be distinguished [82].

There are numerous analytes present in single cells at levels below the limits of detection of many MS systems. On-column and on- or off-line analyte preconcentration methods can be employed to make the analysis feasible. On-line methods are preferable as they avoid analyte loss due to liquid handling or time-dependent degradation. Sample stacking is a commonly used on-column analyte preconcentration method that relies on changes in the electrophoretic mobility of the analytes at the boundary of two buffers prepared at varying concentrations or pH levels. The sensitivity of detection can be improved by 5- to 10-fold using the approach, but its extent is limited by the ratio of the conductivities of the buffers used. Sample stacking was applied in a CE-MS investigation of endogenous nucleotide content of individual *A. californica* neurons [83].

CE-MS technology has reached a level of maturity that allows for investigation of subcellular samples. Aerts et al. [84] examined the metabolite content of different single functioning rat neurons using a patch clamp pipet to collect ~3 pL of cytoplasm from electrophysiologically and morphologically identified thalamocortical neurons, a region known to contain both gamma-aminobutyric acidergic as well as glutamatergic cells. Using CE-MS, approximately 60 metabolites were detected in the cytoplasm of the cells studied. Demonstrating the ability to characterize cells, glutamate was detected in physiologically identified glutamatergic cells, and gamma-aminobutyric acid (GABA) in the cytoplasm of physiologically-characterized GABAergic neurons, while GABA was not detected within the glutamatergic cells. This study demonstrates the usefulness of combining electrophysiological, morphological, and single cell CE-MS approaches for determination of neurochemical correlates of functional activities in neuronal networks.

Microfluidic devices have physical dimensions that are well suited for small volume sample collection, processing, and injection into mass spectrometers. The application of microfluidics in biological research is becoming widespread. Microfluidic platforms allow manipulation of picoliter to nanoliter sample volumes and offer a wide variety of designs and features permitting individual cell capture, culturing, and stimulation, as well as direct sampling of cellular content and/or extracellular microenvironments, analyte separation, and on-line or offline hyphenation to different detection modalities such as MS [33,85]. Preconcentration of analytes can be done directly within devices, e.g., using incorporated solid phase extraction (SPE) materials [86]. As discussed further in the next section, such devices are especially useful for collection, preconcentration, and assaying of analytes released from cells.

5. Measuring cellular microenvironments, including cellular release, via MS

Thus far we have discussed MS approaches and their applications that provide information on the chemical contents of cells [12]. The results of these experiments are useful for the phenotypical classification of cells, but, typically, do not directly reveal functional roles of the detected analytes. In contrast, the investigation of stimulated cellular release provides an opportunity to determine a subset of cellular metabolites and peptides that are likely to be involved in cell-to-cell signaling. Secretomics, the study of the compounds released from a cell, uses a wide range of methodologies, including MS, microfluidics, Western blotting, enzyme-linked immunosorbent assays, gel electrophoresis, radioimmunoassay / labeling with radioactive isotopes, and amperometric detection [79,87–94]. Released compounds vary from small diatomic gases to large proteoglycans and peptides; thus, it is not surprising that a range of characterization approaches are required to assay this large variety of analyte types.

MS is an important tool for studying cellular release as it provides multiplexed information about the protein, peptide, and metabolite content of the releasate without the need for analyte pre-selection. The focus of cellular release studies range from biomarker detection and identification to the determination of protein concentrations [87]. We have chosen to highlight work that has involved the detection and identification of cell-to-cell signaling molecules in the nervous system. Uncovering the peptide composition of cellular releasate

helps to elucidate the functional roles of observed peptides by relating their release parameters to specific stimulation paradigms. For peptides with known activities and targets, it may help to define the physiological role of the studied cell. MALDI-time-of-flight (TOF) MS is often used to study peptide secretion due to its salt tolerance, low detection limits, and ability to be hyphenated with a variety of sample conditioning approaches [14]. As with all single cell studies, secretomics is complicated by the chemical complexity of extracellular environments, low analyte amounts, and the need to remove unwanted chemical components from the sample prior to MS analysis. Microfluidic technologies enable efficient single cell culture, direct cell stimulation, and more specific temporal and spatial capture of released analytes.

Many microfluidic systems have been designed for the capture, treatment, and analysis of single cells, some of which have been applied to investigate analyte release from individual cells or small cellular structures [33,77–79]. Wei et al. [95] developed a series of microfluidic chips that incorporate cell culture and on-chip pretreatment coupled with ESI-quadrupole-TOF MS for analysis. In order to detect glutamate secreted from stimulated PC12 cells, the cells were cultured and stimulated in microchannels on one chip and then the extracellular fluid was moved through tubing to a miniature extraction chip in which the sample was flowed over polymer SPE beads for pretreatment. After pretreatment, the sample was once again moved through tubing to the ion source. This series of chips could easily be used for the analysis of peptides released upon stimulation. Microfluidic devices designed for the detection of peptides are used in biomarker detection assays. Yang et al. [96] presented a device in which specific peptides are affinity captured in microchannels lined with antibodies. This particular system allows detection of as few as 300 peptide molecules and can be when applied in targeted analysis of released peptides.

Collection of the extracellular media and its direct analysis via MALDI-TOF MS is a powerful approach for the analysis of normal and pathological peptide secretion [97]. However, even though MALDI-TOF MS has a high inorganic salt tolerance, the high levels of salts present in the physiological extracellular media hinders effective molecular characterization with MS. Therefore, SPE is often used for sample conditioning as it improves detection, minimizes sample loss, and decreases the salt content before the MS measurement. An analytical system developed by Croushore et al. [98] allowed for the selective stimulation of cultured *A. californica* bag cell neurons through a device in which fluid flow was controlled via applied external pressures and pneumatically controlled microvalves. Once the cells were stimulated, microliter volumes of the cellular microenvironment were collected, purified using SPE, and analyzed by MALDI-TOF MS analysis. This approach allowed low temporal resolution studies of analyte release from low-density cultures and single cells.

Mao et al. [86] studied intercellular communication through the integration of stimulation, release, and pretreatment steps all on one chip. A microfluidic device capable of culturing two distinct cell populations was used for stimulation of one population and a surface tension plug control was used to control the chemical signaling between the populations. Once the desired stimulation occurred, samples of the extracellular media were moved

through the chip and into micro-SPE columns for analyte capture and sample conditioning. After pretreatment, the analysis was performed using ESI-Q-TOF-MS (Fig. 3A).

Quantitative analyses of released analytes can also be performed using a microfluidic platform hyphenated to MS. Zhong et al. [102] developed a device for the quantification of peptides released from *A. californica* bag cell neurons. The device consisted of one serpentine channel treated with octadecyltrichlorosilane (OTS), used to collect the peptides at specific locations. A continuous flow of extracellular fluid brought the released peptides into contact with the OTS. This OTS layer with captured molecules was then interrogated using MALDI MSI. The distance that the peptide was detected along the length of the channel correlated with the amount of the peptide in the sample.

Commercially available, as well as home built SPE devices, are widely used to desalt and concentrate samples prior to analysis via MS. C18 reversed phase media-packed pipet tips have been used for the conditioning of releasates collected from clusters of bag cell neurons of *A. californica* [97], as well as from low-density bag cell neuron cultures [98]. Due to the large volume of C18 material used, this approach can be utilized when release from multiple cells is investigated. However, reducing the volume of SPE material, therefore, leading to higher analyte preconcentration, aids in the detection of the small amounts of analytes released from a single cell. In one example, a small number of SPE particles were mounted onto the surface of Parafilm M. The ~30 μ L volume of collected extracellular media containing cell releasate was deposited onto the particles through a fused silica capillary (Fig. 3B) [97].

Further improvement in the spatial detection of peptide release was achieved by the use of SPE beads placed directly on the processes of cultured *A. californica* bag cell neurons (Fig. 3C) [99]. Alternatively, SPE packed pipette tips or cartridges, as demonstrated by Hatcher et al., can be used to collect peptide release [103]. Finally, to improve both spatial detection and sampling efficiency, lauryl methacrylate-ethylene glycol dimethacrylate porous polymer monolithic columns have been used to investigate both *A. californica* and mammalian systems, attaining sub-picomolar limits of detection with sample volumes of ~ 4 μ L [89].

Additional improvements in analyte retention can be realized by acidification, alkylation, or addition of ion-pairing reagents to the sample. However, in most cases these modifications are not easily applied to the extracellular environment without interfering with the physiological activity of the studied cells. Therefore, to increase SPE analyte extraction efficiency in released peptide analysis, Fan et al. [100] customized particle-embedded monolithic capillaries with pyrrolidone or ethylenediamine in poly(stearyl methacrylate-co-ethylene glycol dimethacrylate) and used them to collect *A. californica* bag cell neuron releasates. The complete system consisted of two capillaries connected to individual syringe pumps, one for application of the secretagogue to the individual neurons at a rate of 0.25 μ L/min and the other for collection and pretreatment of the releasate at the same flow rate for 30 minutes, allowing for low femtomole limits of analyte detection and specific analyte targeting (Figure 3D). This SPE approach was improved in a follow up study [101] with the use of a concentric dual capillary system consisting of an outer capillary that surrounds the stimulation and sampling area, and an inner octadecylmodified silica nanoparticle-filled

capillary to collect and pretreat the ~10 μ L sample. The outer capillary, which delivers the secretagogue, either surrounds the neuron or is positioned above the targeted area of the tissue and remains in that position while the inner sampling capillary can be removed and replaced if multiple collections of releasates are necessary (Fig. 3E). This set-up allows relatively high temporal and spatial resolution of cellular release investigation as well as reduced sample dilution.

6. Concluding remarks

MS-based analysis of small volume samples is a rapidly advancing analytical area. The ability to characterize individual cells and cellular release from selected cells is facilitating several areas of study, including cancer biology, stem cell research, neuroscience and even studies of interactions between specific cells in complex ecological communities. Within the United States alone, there has been a recent emphasis by government funding agencies such as the National Institutes of Health and the National Science Foundation to characterize the cellular makeup of the brain and other organs with single cell resolution.

Of course, more research is needed. After all, genomic and transcriptomic approaches allow the characterization of nearly the complete genome and transcriptome of a selected cell. This is not the case, however, for the metabolomes, proteomes, and peptidomes of single cells, where the fraction of analyte coverage for these limited volume samples is likely only a few percent (or less), especially when examining small cells such as mammalian glia. Small volume sample analysis is rapidly improving its figures of merit with the advent of new sampling protocols, sample conditioning and MS-based approaches, as well as bioinformatics tools to manage the large data sets obtained from these studies. The continued advancement of technologies to monitor individual cells within a complex microenvironment offers great promise for understanding cellular behavior and detecting changes caused by illness, physical damage or exposure to drugs, as well as designing improved therapies for a broad range of diseases.

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Abbreviations

MS	mass spectrometry
MALDI	matrix assisted laser desorption / ionization
MSI	mass spectrometry imaging
MAMS	microarray mass spectrometry
MS/MS	tandem mass spectrometry
ICP-MS	inductively coupled plasma mass spectrometry

ESI	electrospray ionization
TOF	time-of-flight
CE	capillary electrophoresis
SPE	solid phase extraction
OTS	octadecyltrichlorosilane

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Highlights

- Mass spectrometry (MS) is a versatile method for characterizing small volume samples.
- Qualitative, quantitative, targeted, and discovery investigations are enabled by MS.
- Samples as small as individual cells can be assayed for their small molecule content.
- New approaches enable MS-based high throughput small volume sample analyses.

Fig. 1.

Images of the distribution of an analyte at m/z 754.536 from a HeLa cell sample with (A and C) 49 μm and (B and D) 7 μm pixel sizes. The high mass accuracy in (C) and (D) improves image contrast by reducing interference from neighboring peaks (Reprinted with permission from ref [52]. Copyright 2012 American Chemical Society).

Fig. 2. Microarray MS chips allow high throughput sample preparation in single cell analysis. A MAMS chip containing (A) 24 or (B) 12 spots per lane can be automatically aliquoted by dragging the sample over each lane (D–F). (Reprinted with permission from reference [66]. Copyright 2013 American Chemical Society).

Fig. 3.

A variety of SPE-based analyte collection approaches have been developed and applied to small volume sample analysis. (A) An integrated microfluidic device allowing cell stimulation, cell-cell communication, and sample pretreatment. (Reprinted with permission from reference [86]. Copyright 2012 American Chemical Society). (B) Schematic of sample collection from *A. californica* bag cell neurons and its SPE pre-treatment prior to MALDI MS analysis. (Adapted with permission from reference [97]. Copyright 2001 WILEY-VCH Verlag.) (C) Placement of SPE beads (arrows) directly on neurites of cultured neurons. (Reprinted with permission from reference [99]. Copyright 2005 National Academy of Sciences of the United States of America.) (D) A two-capillary system allowing secretagogue application, released compound collection, and sample processing. (Reprinted with permission from reference [100]. Copyright 2011 American Chemical Society.) (E) A concentric dual capillary system used for temporal and spatial investigation of release. Reduced analyte dilution and flexible sample conditioning using octadecyl-modified silica nanoparticles are important properties. (Reproduced from reference [101] with permission from the Royal Society of Chemistry.)