Development of Imaging Mass Spectrometry

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Received February 3, 2012

We have developed a mass microscope in which a microscope is combined with high-resolution matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS). This technique is a powerful tool for investigating the spatial distribution of biomolecules without the need for any time-consuming extraction, purification, and labeling procedures for biological tissue sections. The mass microscope provides clear images with regards to the distribution of hundreds of biomolecules in a single measurement, and also helps in determining the cellular profile of the biological system. In this review, we focus on some of the recent developments in clinical applications and describe how the mass microscope can be employed to assess pathomorphology and pharmacokinetics.

Key words imaging; mass spectrometry; microscopy; analytical biochemistry; drug kinetics

1. INTRODUCTION

Imaging is a technique that can be used to visualize cellular and molecular processes that occur in living organisms in a two-dimensional (2-D) or three-dimensional (3-D) fashion, without perturbing the structure of the system. The various techniques currently available include X-rays,1) nuclear magnetic resonance,2) cryo-electron microscopes,3) positron emission tomography,4) immunohistochemistry,5–8) green fluorescent protein labeling,9–12) and luciferase.13) The green fluorescent protein labeling technique involves over expression of the fusion protein of the concerned molecule and green fluorescent protein.12,14) However, these techniques can only provide information on the structure of the material. There is also a “nonlabeling” technique even at the electron microscopic level,15) and yet there is still a serious limitation on the object preference. Imaging mass spectrometry (IMS)16) is an emerging technique that is expected to at once resolve these problems in conventional morphological examinations. In IMS, mass spectra associated with spatial information can be simultaneously recorded to obtain expression patterns of various molecules in specimens to be analyzed. This new generation of mass spectrometry (MS) has been used for the analysis of biological compounds at either the tissue6–19) or single-cell level.20–23) Recent IMS studies have been conducted on a variety of topics, including biological applications24–30) and pathological applications.31,32) In addition to the analysis of protein described in the above references, direct lipid analysis in mammalian tissues33,34) has also been conducted, and histopathological materials35) and pharmacokinetics in rat whole body sections36) have been studied.

IMS can be used to visualize the spatial distribution of biomolecules based on the mass-to-charge ratio (m/z) of the target molecule in the mass spectrum. Several ionization methods, such as desorption electrospray ionization (DESI),17) secondary ion mass spectrometry (SIMS),38) and matrix-assisted laser desorption/ionization (MALDI) have been investigated.39) DESI is an ionization technique by which the molecules are ionized without addition of organic matrix under ambient conditions. In this method, the surface of the sample is analyzed by charged droplets of solvent, generated during the electrospraying. DESI has a limited spatial resolution of 0.3–0.5 mm, which is not a sufficiently high resolution for imaging. SIMS on the ion microprobe offers the best combination of spatial resolution (20 μm beam diameter) and precision (0.2 per mL for sulfur isotopes). This technique is applicable for the analysis of small molecules (<1 kDa) because the high energy of the SIMS causes the fragmentation of larger molecules. On the other hand, MALDI is used to visualize biomolecules such as lipids less than 1 kDa as well as peptides and proteins over 10 kDa.

MALDI-IMS is a powerful tool that allows simultaneous mapping of hundreds of molecules in a tissue section in a single measurement. To use a mass spectrometer as an imaging instrument, it is essential for the spectrometer to be equipped with an automatic rastering function, automatic data acquisition system, and visualization software. Recently, several manufacturers have released novel instruments having these features. Almost all manufacturers have developed in-house software for their instruments and have included a driver for instrument and image reconstruction. On the other hand, the user-friendly visualization software BioMap40) is freely available; it is used commonly in mass imaging (MALDI MSI HP, http://www.maldi-msi.org/content).

Our group has developed the original equipment in collaboration with Shimadzu Corporation of Japan and extended the techniques for molecular profiling of different tissue samples, such as brain,41–43) liver,44) testis,45) and retina46) of mice, and colon cancer in human,47) involving sample preparation,25,48–50) and the nanoparticle-based ionization process in IMS.51–53)
recent years, MALDI imaging has resulted in many developments for assessing the localization of molecular species in biological samples. Several applications represent the direct entailment of this technology to basic clinical research. Here, we discuss the recent developments concerning MALDI-IMS.

2. TARGETS OF ANALYSIS BY MALDI-IMS

2.1. Imaging of Glycosphingolipids Glycosphingolipids (GSLs) play important roles in various brain functions. To investigate the mechanisms of brain function in more detail, it is necessary to understand the composition and function of GSLs. We have studied GSLs in previous research.56–58 Thin-layer chromatography (TLC) is routinely used for the separation and partial characterization of neutral and acidic GSLs and phospholipids in mixtures.56–58 However, even under optimized TLC conditions, TLC characterization of individual GSLs does not yield unambiguous structural information. Information regarding the compositions of these highly complex mixtures remains limited. The same sugar moiety in different GSLs may migrate to different positions owing to differences in their ceramide structures. Multi-stage MS (MS^n) analysis can supply information on each ceramide structure. However, it is difficult to identify individual molecular species by MS alone. Therefore, by combining TLC and MS, we were able to obtain a complete set of information on GSLs.

Our group has used MALDI-MS/MS to study colon cancer liver metastasis in 3μm thick tissue sections. MS/MS investigations of normal and cancerous cells revealed
that the cancerous cells accumulated sphingomyelin.\(^{47}\) We have also studied the localization of seminolipids in mouse testis during testicular maturation, and the organ-specific distribution of lysophosphatidylcholine and triacylglycerol in mouse embryos using IMS.\(^{45,59}\) Recently, our group has developed a TLC-Blot-MALDI-TOF-IMS system which can separate and partially characterize acidic and neutral GSLs. Here, we describe gangliosides identified from a control patient and a patient with Alzheimer’s disease using TLC-Blot-MALDI-TOF-MS imaging of the hippocampus gray matter (Fig. 1). In the Alzheimer’s patient, the GM2 and GD3 ganglioside bands appeared to be clearer than those of the control. The relative increases in d18:1 sphingosine-containing gangliosides in the patient with Alzheimer’s disease are also expressed in this image.\(^{60}\)

### 2.2. Imaging of Proteins and Peptides

Immunohistochemistry has been commonly used for profiling protein distribution in tissue sections. In this approach, antibodies are needed to detect specific proteins. Other genomic and proteomic approaches cannot be applied to biopsies as the quantity of sample available is only very small.\(^{14,61–63}\) Our group has analyzed SCRAPPER (a protein we first reported that is localized in neuronal synapses,)\(^{6}\) knockout mouse by utilizing a proteomic approach based on an IMS technique.\(^{64}\)

We have also demonstrated that the denaturation process and detergent-supplemented trypsin solution can improve the protein digestion efficiency for direct tissue analysis with IMS.\(^{65}\) We recently developed a formalin-fixed paraffin-embedded

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**Fig. 3. IMS and Histology of the Samples**

We constructed a figure by plotting the positions of the m/z 782.5, 1074.6, 1102.6, 1130.7 and 1156.7 peaks. When we compared (A) IMS with (B) a hematoxylin and eosin-stained section, the peaks of Gb3s were more densely packed in cardiomyocytes with vacuolar degeneration, and the Gb3s existed together in some parts and separately in others. (C, D) In contrast, Gb3 was not detected in a control sample from a patient with secondary myocardial degenerative changes with aortic regurgitation. (E, F) Gb3s were also detected on IMS in the heart of a mouse with Fabry’s disease even though there was no evidence of vacuolar changes in the myocardium on light microscopy. Bar, 200\(\mu\)m. (Reprinted from ref. 67 from Official Journal of the Japanese Circulation Society.)
tissue microarray to study gastric carcinoma tissue samples by IMS, and successfully identified histone (H4)-specific signals in poorly differentiated cancer tissue samples by utilizing tandem MS.\(^6\) Proteomic-based IMS investigations have resulted in a better understanding of carcinogenesis, invasiveness, metastasis, and the prognosis process in gastric cancer patients.

3. DEVELOPMENT AND APPLICATION OF HIGH-RESOLUTION ATMOSPHERIC PRESSURE-LASER MASS MICROSCOPE

3.1. Development of a ‘Mass Microscope’

Recently, we have developed a ‘mass microscope’ consisting of a microscope coupled with high-resolution atmospheric pressure-laser desorption/ionization (AP-LDI) and a quadruple ion trap-time-of-flight (QIT-TOF)-analyzer\(^6\) (Fig. 2). This instrument allows us to precisely observe a specific tissue section before IMS and analyze the biomolecules with a spatial resolution of 10\(\mu\)m on the tissue section. An UV laser tightly focused with a triplet lens was used to achieve high spatial resolution. An atmospheric pressure ion-source chamber enables us to analyze fresh samples with minimal loss of intrinsic water or volatile compounds.

3.2. Analysis of Disease Biomarkers in Fabry’s Disease

We have used IMS to achieve the accurate diagnosis of Fabry’s disease, especially in questionable cases, and have shown that IMS has a higher specificity than electron microscopy or enzyme activity assays, which are based on light microscopy. We constructed images by determining the locations of these peaks within an endomyocardial (EMB) biopsy sample.\(^6\) We observed that the distribution of globotriaosylceramides (Gb3s) was consistent with that of cardiomyocytes, especially in areas that were affected by vacuolar degeneration, and that the Gb3 types existed together in some parts and separately in others (Figs. 3A, B). In contrast, as shown in Figs. 3C and D, Gb3 was not detected in the control EMB sample. When we analyzed the heart from a mouse with Fabry’s disease, we also detected Gb3s in the cardiac tissue, even though there was no evidence of vacuolation in the cardiomyocytes on light microscopy (Figs. 3E, F). Gb3 was not detected in the control mouse heart. We could detect Gb3s not only in the heart from a Fabry’s disease patient, but also in the heart from a mouse model of Fabry’s disease without discernible degenerative changes on light microscopy. Although the significance of each type of Gb3 distribution is unknown, it is possible that these distribution patterns could help to distinguish variations in the disease phenotype or evaluate the effectiveness of enzyme replacement therapy, which may also help to elucidate the basis for the disease. However, this issue requires further study. The current study presents novel findings suggesting that IMS is useful for diagnosing Fabry’s disease with cardiac manifestations, especially in questionable cases. Because IMS can directly analyze the molecular weight of each existing component, IMS has a higher specificity than electron microscopy or enzyme activity assays when Fabry’s disease is suspected based on light microscopy. Our results indicate that IMS is a new tool that can be used to accurately diagnose not only Fabry’s disease, but also other unknown storage diseases.

3.3. Analysis of Biomolecules in Human Hair

The mass microscope has a subcellular spatial resolution of 10\(\mu\)m for the detection of molecules from a tissue section. We have

Fig. 4. Hair-Specific Mass Spectra of Putative Aging Markers

ROI-specific mass spectra in subject No. 1 are presented. Red peaks and blue peaks are derived from the hair section and background area, respectively. (A) ROI selection is illustrated: ROI A as hair section and ROI B as background area. (B) m/z 50 to 300. (C) m/z 125.99. (D) m/z 153.00. (E) m/z 164.00. (F) m/z 207.04. (Reprinted from ref. 68 with permission from The Public Library of Science.)
Fig. 5. Mass Spectrum of Famotidine and an MS Ion Image of a Whole-Body Section

(A) Structure and mass spectrum of famotidine. (B) Mass spectrum of famotidine detected in a tissue section. (C) Mass spectrum of famotidine detected in the kidney in a tissue section. (D) Optical image of a mouse tissue section. (E) MS ion image of famotidine (m/z 338.05) on a mouse tissue section 3 min after injection. Famotidine is localized to a significant extent in the kidney. (F) MS ion image at m/z 338.05 on a control mouse tissue section. (Reprinted from ref. 69 with permission from Journal of the Mass Spectrometry Society of Japan.)
applied this system to analyze human hair cross sections, and have detected biomolecules showing aging-related alterations. The optical images obtained at high resolution showed molecular distribution in the cortex and medulla region of hair. Among the 31 molecules detected specifically in hair sections, dihydrouracil and 3,4-dihydroxymandelic acid (DHMA), which are metabolites of uracil and catecholamines, respectively, exhibited a higher signal intensity in the young group than in the old, and O-phosphoethanolamine displayed a higher intensity in the old group (Fig. 4). Among the 3, putative O-phosphoethanolamine showed a cortex-specific distribution, and exhibited changes in signal intensity with aging, whereas the molecules in medulla did not exhibit significant changes.

3.4. Analysis of Drug Distribution Imaging of drugs and metabolites by MALDI-IMS offers a unique opportunity to identify changes in the distribution of a desired compound in different regions of a tissue of interest, and this technique can help us to understand whether an exogenous compound administered orally affects endogenous metabolites. Antipsychotic, cancer, anti-anxiety, and hypnotic drugs have been studied in different tissue sections by IMS to determine the distribution of molecules. In these assays, a multivariate solution analysis revealed that the distribution of famotidine is concentrated in the renal pelvis through the corticomedullary axis. The results suggest that famotidine is more concentrated in the renal pelvis of the kidney. The results suggest that famotidine is more concentrated in the renal pelvis and released by insulin stimulus in adipocytes. "J. Biol. Chem.," 281, 39273–39284 (2006).

4. FUTURE PERSPECTIVES Many great advances have been made in MALDI-IMS to resolve molecular species in various types of biological samples, but there is still room for improvement with respect to sample preparation, ionization, and instrumentation. The mass microscope could be a powerful tool for obtaining high resolution of biomolecules in tissue samples. The fundamental contributions of MALDI-IMS will provide a powerful tool for the early detection and characterization of cellular processes in both healthy and disease conditions, and help us to understand and treat disorders very effectively.

Acknowledgments This review was undertaken on behalf of The Pharmaceutical Society of Japan for Awarding the Promotion Prize to M.S. for the development and application of IMS techniques and mass microscope. We would like to acknowledge the collaboration of past and present colleagues in the Department of Cell Biology and Anatomy of Hamamatsu University School of Medicine, Shimadzu Corporation, and many other collaborators for their work that was reviewed in this paper and supported mainly by the Japanese Science and Technology Agency (JST) in the form of a Grant-in-Aid for SENTAN.

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