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REVIEW ARTICLE

MALDI imaging: beyond classic diagnosis

Laura Denise Manzanares-Meza[a,b], **Claudia Ivonne Gutiérrez-Román**[a,c], **Oscar Medina-Contreras**[a,*]

[a] *Laboratorio de Inmunología y Proteómica, Hospital Infantil de México Federico Gómez, Mexico City, Mexico*
[b] *Departmento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados, Mexico City, Mexico*
[c] *Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico*

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Abstract Mass spectrometry has been the focus of technology development and application for imaging for several decades. Imaging mass spectrometry using matrix-assisted laser desorption ionization is a new and effective tool for molecular studies of complex biological samples such as tissue sections. As histological features remain intact throughout the analysis of a section, distribution maps of multiple analytes can be correlated with histological and clinical features. Spatial molecular arrangements can be assessed without the need for target-specific reagents, allowing the discovery of diagnostic and prognostic markers of different cancer types and enabling the determination of effective therapies.

PALABRAS CLAVE
Espectrometría de masas;
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Imagenología por MALDI: más allá del diagnóstico clásico

Resumen La espectrometría de masas ha sido el foco de atención del desarrollo y aplicación de tecnología para imagenología por varias décadas. La imagenología por espectrometría de masas utilizando ionización por desorción láser asistida por matriz es una herramienta novedosa y efectiva para el estudio molecular de muestras biológicas complejas como los cortes de tejidos. Ya que las características histológicas se mantienen intactas durante el análisis de

* Corresponding author.
E-mail address: omedina@himfg.edu.mx (O. Medina-Contreras).

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una sección, pueden correlacionarse mapas de distribución de múltiples analitos con características clínicas e histológicas. Los arreglos moleculares espaciales pueden evaluarse sin la necesidad de reactivos específicos para el blanco, lo que permite el descubrimiento de marcadores pronósticos y diagnósticos de diferentes tipos de cáncer y permite la determinación de terapias efectivas.

1. Introduction

To gain a full understanding of cancer biology and to be able to translate this information to the clinic requires substantial information about the proteome through the course of a disease. Improved technology means the generation of vast amounts of information about the cancer proteome. Proteomics-based techniques allow the identification of biomarkers and protein expression signatures, which could be used to predict responses to drugs and the clinical course of a disease, and such information could be used to individualize therapy. Indeed, proteomics and imaging technologies are being combined to improve diagnostics, histological grading of tumors, and responses to therapy. Such progress is complemented by the numerous clinical samples gathered in biobanks, which allows researchers to screen large cohorts and validate their findings. Also, proteomics techniques are being used to gain an understanding of how signaling pathways are altered in tumor cells so that the underlying biology of a human tumor can be understood. Moreover, proteomics platforms have been enlisted in drug development to identify new drugs and to improve our understanding of how to target various pathways.

Mass spectrometry (MS) has become an invaluable tool for the characterization of proteins and can be used to measure the molecular weights of intact polypeptides, determining peptide identity, and delineate peptide structure. Identification of biochemically isolated proteins that are represented in databases, either as translations of polynucleotide sequences or as independently determined amino acid sequences, may be the largest current application of mass spectrometry in the emerging field of proteomics.

Imaging MS (IMS) using matrix-assisted laser desorption ionization (MALDI) is a specialized and powerful tool to understand the spectrum of many compounds expressed in intact tissue sections, such as lipids, proteins/peptides, and metabolites.

MALDI-IMS has several advantages over traditional techniques, such as classical histology, autoradiographic methods, or tissue homogenates analysis. MALDI-IMS has minimal impact on histological features, is a label-free spatial analysis that allows the rapid discovery of disease-associated markers, and has the potential to identify specific tissue location of parent drugs and its metabolites. Thus, the distribution of multiple compounds can be correlated with histological and clinical features, improving the ability to propose new therapeutic treatments. This review will focus mainly on the application of MALDI-IMS for the analysis of tissue samples in the clinical setting. Several reviews provide a broad overview of other MS imaging methods and applications.[1–7]

2. Overview of MALDI-IMS

MALDI-IMS is a versatile technique used to understand molecular mechanisms in biology and medicine, particularly in the development and progression of cancer. MALDI-IMS analyses can detect and locate hundreds or more specific signals from endogenous biomolecules (metabolites, proteins, peptides, lipids) and exogenous molecules (drugs) simultaneously in intact tissue sections. This simultaneous detection provides an invaluable opportunity to evaluate the underlying physiological state of a tissue.

However, sample handling and preparation are crucial for achieving high quality and reproducibility in MALDI-IMS studies. Typically, thin tissue sections are coated with an energy-absorbing matrix to extract analytes of interest from the underlying tissue and co-crystallize the extracted molecules with the matrix, which are subsequently irradiated with a laser beam, generating either singly protonated or deprotonated molecular ions that are detected by a mass analyzer and separated based on their molecular mass to charge ratio (m/z). Mass spectra are acquired across the tissue at defined geometrical coordinates, and specific algorithms allow data analysis and classification, protein identification, and generation of three-dimensional images of tissue sections. This approach allows the correlation between mass spectra and anatomical features.

3. Applications in clinical research

Histology allows the identification and localization of specific antigens on tissue sections. Histopathological examination of tumor biopsies allows diagnosis confirmation and tumor classification. However, immunohistochemistry (IHC) and immunofluorescence (IF) microscopy rely on antibodies for antigen detection, limiting its application. MALDI-IMS is capable of multichannel label-free identification, generating a comprehensive spectrum of peptides/proteins expressed in tissue sections. These data provide clinically relevant information beyond the current limitations of histopathological techniques. This tissue-based spatial proteomic approach enables studies of protein abundance and distribution, as well as to identify regionalization of these proteins. Thus, MALDI-IMS enhances the detection power of traditional histology through the simultaneous identification

of multiple mass spectra of peptides. As it will be reviewed in the following sections, MALDI-IMS data provides a powerful tool in cancer biology, histology, and the identification of novel biomarkers.

3.1. Tumor classification

Clinical diagnosis and patient stratification rely on the correct identification of the nature and origin of a metastatic tumor, especially when no primary tumor is detected. Current methods involve clinical and histological techniques, while molecular classification focuses almost exclusively on genetic mutations. MALDI-IMS has been successfully applied to diagnostic to differentiate between tumor and normal tissue, or different tumor subtypes.

Using MALDI-IMS, correct tumor classification has been achieved using tissue-based proteomics. Proteomic signatures obtained from various tumors allowed the identification of adenocarcinomas from different organs with accuracies above 80%. Further, the proteomic signatures obtained were able to discriminate hepatocellular carcinoma from colon cancer liver metastasis with high accuracy.[8] Melanoma lymph node metastases were also discriminated from disease-free lymph nodes, and molecular subclassification was achieved in patients with stage III disease. Further, combined mass spectra generated multiplex molecular signatures to group patients into either poor or favorable groups for recurrence and survival.[9] Non-small cell lung cancer correct subtyping into adenocarcinoma or squamous cell carcinoma is fundamental for proper treatment. Formalin-fixed paraffin-embedded (FFPE) tissues were analyzed by MALDI-IMS, and proteins selectively expressed were identified and validated by IHC. Tissue classification correlated above 99% with pathological diagnosis and revealed less known differentially expressed proteins.[10] Finally, hepatitis B virus (HBV)-related hepatocellular carcinomas (HCC) tumors were differentiated based on a progressive regional change in protein abundance and distribution from non-tumor to tumor regions.[11] Aberrant protein expression is an important hallmark of several types of cancer. HER2-testing is mandatory in breast and gastric cancers for proper treatment with the monoclonal antibody trastuzumab. HER2 is a known marker of breast and gastric cancer, and based on the proteomic profile of a breast cancer classifier a HER2 prediction model was established, able to predict HER2-status in gastric cancers with a high sensitivity and specificity, suggesting that HER2 overexpression may constitute a unique molecular signature independent of the tumor site, identifiable by IMS.[12]

FFPE tissue samples are the gold standard in histopathology; however, it was believed that mass spectra information changed during tissue processing. A novel method for high-throughput proteomic analysis of FFPE tissue microarrays (TMA) was developed using MALDI-IMS. A lung tumor TMA was analyzed, and mass spectra was correlated to defined histological regions in serial hematoxylin and eosin (HE)-stained sections. The statistical classification models generated differentiated adenocarcinoma from squamous cell carcinoma,[13] similar to non-FFPE tissue.

Together, these findings demonstrate high accuracy MALDI-IMS-derived proteomics classification is a valuable tool to discriminate between different tumor types at different organs and in the same site. The ability to detect and characterize tumor marker proteins for large cohorts of FFPE samples provides a high-throughput approach for diagnostic and prognostic purposes.

3.2. Biomarker identification

There is a pressing need to identify disease-specific molecular markers in biopsy tissue to improve diagnosis and treatment. MALDI-IMS has been successfully used for the identification of proteins that predict disease outcome. Studies using MALDI-IMS have revealed differences in the proteomics profiles of healthy and cancer tissues, and have characterized new diagnostic and prognostic biomarkers in gastric cancer,[14] hepatocellular carcinoma,[15] Hodgkin lymphoma,[16] breast cancer,[17] and ovary carcinoma,[18] among others.

Protein profiles from intestinal-type primary resected gastric cancer tissues revealed a seven-protein signature associated with an unfavorable overall survival independent of major clinical covariates. Further validation of the prognostic significance of individual proteins identified a protein previously unknown in gastric cancer and confirmed it as a novel and independent prognostic factor for all patients in a validation cohort, which established a new independent indicator of patient survival complimentary to the previously known clinical parameters regarding prognostic relevance.[14] A comparison of the proteome of HCC and peritumoral cirrhosis from frozen liver tissues identified a set of proteins with a differential intensity level that most accurately delineated cancer from an adjacent cirrhotic tissue. Validation of these results matched histological analysis and identified monomeric ubiquitin as the protein most overexpressed in HCC, which was validated by tissue microarrays, suggesting a new tool for diagnosis of difficult HCC and identification of candidate biomarkers.[15] Proteome analysis of Hodgkin lymphoma led to the identification of differentially expressed proteins able to distinguish between classical Hodgkin lymphoma and lymphadenitis, with a sensitivity and specificity above 80%.[16]

HER2 status determination directly in tissues identified specific protein/peptide expression changes that correlated with HER2 overexpression, generating a proteomic signature able to define accurately HER2-positive from HER2-negative tissues.[17] Prostate cancer (PCa) samples analyzed by MALDI-IMS revealed a protein profile capable of discriminating cancer from normal tissue,[19] and at different times during normal prostate development.[20] Further, this discrimination was possible with a single peptide, and its identification revealed a novel marker of PCa. Finally, a biomarker with high prevalence was identified by comparing stages III and IV ovary carcinoma tissues to healthy ovaries. Validation of this marker performed by classical immunocytochemistry, MALDI-IMS, and Western blot analysis confirmed changes in its localization from the nucleus in benign epithelial cells to the cytoplasm in carcinoma cells.[18]

Together, these studies highlight the usefulness of MALDI-IMS in generating specific proteomic signatures associated with cancer establishment and severity.

3.3. Molecular histology

Histopathology is the standard approach for tissue diagnostics, and the gold standard in histology are FFPE tissues. However, metabolite evaluation in these tissues has not been performed routinely due to concerns about changes in metabolite content or chemical state due to tissue processing. Using Fourier-transform ion cyclotron resonance MALDI-IMS, a novel protocol for the *in situ* analysis of metabolites in formalin-fixed paraffin-embedded was developed. Using this platform ~1,500 mass spectra were identified, with more than 70% overlap with fresh frozen samples. Further, this protocol was successfully applied to tissue microarrays and biopsies. Thus, metabolites are largely conserved in FFPE tissue samples.[21] Recurrence after tumor resection suggests molecular changes in surrounding histologically normal tissue go undetected by conventional diagnostic methods. The molecular distribution within a tumor and adjacent normal tissue in clear cell renal cell carcinoma biopsies revealed that normal tissue adjacent to the tumor express several tumor molecular markers, indicating the ability to elucidate aberrant molecular changes in the tumor microenvironment by MALDI-IMS.[22] Myxofibrosarcoma and myxoid liposarcomas are common soft tissue tumors with histological overlap. A study on 40 well-documented myxofibrosarcoma and myxoid liposarcoma cases using IMS detected tumor specific proteins and lipids and revealed lipid changes related to tumor progression. Tissue classification was consistent with pathological classification, but also revealed intratumoral heterogeneity associated with nodular structures.[23]

Changes in the proteomic profile during wound healing is critical for the establishment of the molecular mechanism involved in burn injury. A study on the normal and healing skin from patients with burn wounds examined the spatiotemporal protein profile and identified expression of inflammatory protein markers early after injury. These proteins decreased as healing progressed, revealing part of the molecular sequences associated with the systemic inflammatory response following burn trauma.[24] Opioid peptides are associated with different neuropathologies. Prodynorphin has been associated with L-DOPA-induced dyskinesia (LID) at the mRNA level, but not at the protein level. Using MALDI-IMS the characterization, localization, and relative quantification of neuropeptides was performed in a rat model of LID in Parkinson's disease. Several peptides were detected at higher levels than controls, and the intensity correlated with the disease severity, notably dynorphin peptides, providing a tool for the study of molecular dynamics in diseases where other tools are not available.[25] Multiple sclerosis is characterized by recurrent inflammatory demyelinating lesions. A recent pilot study identified peptides expressed in multiple sclerosis brain lesions and classified the lesions similarly to histopathological analysis. Further, in-depth mass spectra analysis revealed lesions not detected by IHC and identified thymosin beta-4 distributed at the edge of these lesions, which was later validated by IHC.[26]

Lipid imaging has proven difficult through traditional methods. Phosphatidylcholine (PC) is the most abundant component of lipid bilayers, and some of its molecular forms are fundamental in neuronal signaling. Using MALDI-IMS PC intracellular distribution in neurons of the superior cervical ganglia was imaged revealing a proximal to a distal gradient, which is dependent on actin dynamics.[27] Sulfatide an extremely important sphingolipid in the nervous system. Sulfatide can exist in hydroxylated and non-hydroxylated forms, which influence myelin sheath stability. The identity and localization of 14 sulfatide species in cerebral cortex tissue sections by IMS revealed a different ratio in hydroxylated and non-hydroxylated forms between white and gray matter. Moreover, this distribution is not affected in neurodegenerative diseases such as Alzheimer's disease.[28] Similarly, gangliosides are important for memory formation, neuritogenesis, and synaptic transmission in the nervous system. C18- and C20-sphingosine molecular forms were found differentially expressed in the brain, regulated specifically during development and aging.[29] Testosterone, a cholesterol-derived androgen, and seminolipid, an ether glycerolipid, are fundamental for testis development and spermatogenesis. Testosterone was imaged in human chorionic gonadotropin (hCG)-treated testis inside and outside the seminiferous tubules. MALDI-IMS revealed a 228-fold increase compared to control tissues, consistent with the quantitative analysis that showed a 256-fold-increase in hCG-treated tissues. Further, IMS high-spatial resolution confirmed testosterone accumulates on Leydig cells.[30] Similarly, seminolipid molecular species were differentially expression in a cell-specific manner during testicular maturation.[31]

3.4. Metabolism

Cancer establishment is often preceded by changes in cell metabolism. Therefore, the detection and spatial localization of metabolites may help to understand the metabolism of cancer. An altered metabolism in brain tumors has been identified long ago, using MALDI-IMS a metabolic shift to aerobic glycolysis was imaged in glioblastoma multiforme, and a mutation in the IDH1-gene, as well as the distribution of chloride anions involved in growth of glioma tumor cells, were identified in specific regions within the tumors.[32] Aortic atherosclerotic lesions also allowed the visualization of specific markers on aortic roots of apolipoprotein E-deficient mice and femoral arteries of humans with peripheral artery occlusive disease. Several molecular ions were found to be specific molecules with similar distributions in the mouse and human lipid-rich regions, revealing lipid-rich and calcified regions in the atherosclerotic lesions.[33] Inflammation associated with abnormal lipid metabolism is associated with the development of varicose veins (VV). To elucidate the lipid metabolism of VV MALDI-IMS was performed in tissue from patients who underwent great saphenous vein stripping or peripheral artery occlusive disease who underwent infra-inguinal bypass with reversed saphenous vein grafting. A unique pattern of lipid molecules in the VV tissues was identified, with lysophosphatidylcholine,

phosphatidylcholine, and sphingomyelin specifically localized at the site of the VV valve.[34] Finally, changes in the lipid profile in intestine in response to different diets were also identified.[35] Imaging of drugs and its metabolites can provide a quantitative method in cells and tissues. A novel method identified the localization of HIV protease inhibitors and allowed its quantitation at clinically relevant concentrations.[36] The mechanism of skin blanching associated with topical glucocorticoids is not fully understood. Three novel glucocorticoids evaluated for their degree of percutaneous absorption showed differences in their potency and time course compared to *in vitro* experiments. MALDI-IMS identified a correlation between the depth of skin penetration and the degree of skin blanching, which explained the different responses.[37] Energy metabolism has also been evaluated by imaging energy-related metabolism. The distribution patterns of ATP, ADP, and AMP in the hippocampus were identified, and a reduction in ATP and ADP levels, but not AMP, during a seizure was observed, indicating a spatiotemporal neuron selective energy metabolism together with accelerated glycolysis during seizure.[38]

During drug development is critical to determine the distribution and metabolism of therapeutic compounds. Studies with drugs or monoclonal antibodies in kidney, liver, lung and glioma showed that MALDI-IMS has much higher spatial resolution than traditional imaging techniques, such as positron emission tomography, magnetic resonance imaging, and whole-body radiography.[39] These studies also highlight the specific high-resolution identification of unlabeled compounds at sites of *in vivo* uptake and retention.[40]

3.5. Development

Embryo implantation requires a complex and unique interaction between the uterus and the blastocyst. *In situ* proteome[41] and phospholipid[42] profiles of implantation were generated from uterine sections. The complex region- and stage-specific molecular composition, relative abundance, and spatial distribution of proteins and phospholipids were analyzed during the progression of implantation by MALDI-IMS, revealing a complex and dynamic network of distribution and interactions in early pregnancy.

3.6. Other applications

Although this review is focused on clinical research, MALDI-IMS has been successfully implemented for other different application areas including xenobiotics, microbiology, pharmaceutical research, model organisms, plants, and DNA analysis.

4. Concluding remarks

MS-based imaging approaches in biology and medicine provide a great analytical depth into the structure and function of tissues and biomolecular systems. While the information generated is complementary to traditional biochemical or histological approaches, often it provides much more evidence to understand the highly complex and dynamically interacting biomolecular mechanism.

The *in situ* identification of molecular signatures of distinct and specific peptides for different tumor regions provides a powerful tool that may offer better classification and prognosis of cancer. The ability to detect and characterize tumor marker proteins in a systematic way offers a high-throughput approach for histology-based applications. This allows a detailed study of the architecture of tissues and organs, and a focus on integrated structural studies which provide a global picture of key cellular components.

Evidently, MALDI-IMS label-free approach offers a clear advantage over traditional techniques. This feature allows the study of molecules for which there are no biological reagents available. Moreover, the ability to perform multiple spatial detections in a single slide leave more tissue sample for further molecular analysis. Together, data summarized in this review highlights the importance of MALDI-IMS in diagnostic and prognostic for both researchers and clinicians; this may be translated into an improved patient outcome.

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Conflict of interest

The authors declare no conflicts of interest of any nature.

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