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6

Nanoparticle-assisted Laser Desorption/ionization (nano-PALDI)-based Imaging Mass Spectrometry (IMS) and its Application to Brain Sciences

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ABSTRACT

Imaging mass spectrometry (IMS; also referred to as mass spectrometry imaging [MSI]) is an emerging mass-spectrometry-based imaging technique that enables visualization of the distribution of various biomolecules in biological tissue sections. This technique, which can be used for a variety of tissues

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List of abbreviations after the text.

having vast structures, was initially developed as a tool for protein imaging. However, because of the general versatility of IMS and the lack of established imaging technology for small organic molecules, the number of studies reporting IMS of small molecules has recently increased. In fact, IMS is an effective technique for the visualization of endogenous small metabolites, especially lipids, facilitated by the unique advantages of mass-spectrometry-based molecular detection. For IMS, the choice of a proper analyte ionization technique is critical. Matrix-assisted laser desorption/ionization (MALDI) has been regarded as the most effective analyte ionization method and has been applied to the analyses of brain disorders, such as Alzheimer's and Parkinson's diseases. Despite the promising capability of MALDI-based IMS for imaging of small metabolites, this technique suffers from several critical drawbacks, especially with regard to spatial resolution. One of the critical limitations of the spatial resolution of MALDI-IMS is the size of the organic matrix crystal and analyte migration during the matrix-crystallization process. To overcome these problems, we report herein a nanoparticle (NP)-assisted laser desorption/ionization (nano-PALDI)-based IMS technique, in which NPs are used as the ionization-enhancing reagent and the organic matrix crystallization process is eliminated. Another important advantage of the use of NPs for IMS comes from the recently increasing availability of various NPs with different core-metals, surface modifications, and particle diameters, which has expanded the range of molecular species that can be analyzed by means of this technique, to include species that cannot be ionized by MALDI-IMS. Hence, we believe that this new approach will lead to a better understanding of physiological processes as well as the diagnosis and pathophysiology of complex biological process, especially in the brain. This chapter summarizes the recent technological developments in the field of IMS and also describes the utilization of nano-PALDI in IMS as an attractive alternative to traditional MALDI-IMS.

INTRODUCTION

In the last decade, the practical use of nanoparticles (NPs) in the field of biomedicine, particularly as nanomachines, molecular imaging probes, biosensors, diagnostic tools, and drug-delivery systems, has been reported extensively. With the goal of improving the therapeutic efficacy of drug-delivery systems, NPs, which are small-sized particles having diameters

in the range of 100–1000 nm, have been frequently exploited as carriers for macromolecules [e.g., plasmid DNA, siRNA, peptides, and genes] and small molecules [e.g., a corticosteroid and alkaloids]. The details of these applications have been described in a recent review by Taira et al. (2009). NPs have also found practical application in mass spectrometry (MS) research.

Historically, one of the currently employed "*standard*" soft ionization techniques in MS, matrix-assisted laser desorption/ionization (MALDI), was initiated by Tanaka's study, which was awarded the Nobel Prize in Chemistry; in this technique, a suspension of an inorganic NP such as cobalt, silicon, or titanium nitride was utilized as an analyte ionization enhancing reagent, referred to as a matrix (Tanaka et al. 1988). Although subsequent studies have successively developed various series of organic matrix compounds, which are currently the most frequently used matrices (Karas and Hillenkamp 1988), NPs have again begun to attract increasing attention owing to the progress in technology, which has resulted in the production of a wide variety of NPs. The increasing availability of various kinds of NPs, especially with variations in sizes, core materials, and surface coating/chemical modifications, has allowed researchers the option of choice from a selection of NPs to suit the chemical/physical requirements of their research purposes. In fact, the use of NPs as an alternative to organic matrices in MS research has been widely studied, particularly in the analyses of small molecules. In this regard, the use of NPs offers a number of attractive advantages including the elimination of noise generated by the organic matrix compounds. In addition, based on the proper choice of core metal material and surface modification, analyte molecules that are difficult to ionize using conventional organic matrices can be very efficiently ionized using NP matrices (Su and Tseng 2007).

This chapter describes the application of NPs to the emerging MS methodology known as imaging mass spectrometry (IMS). In IMS, which is an MS based molecular imaging technique, distributions of analyte molecules are visualized from the mass spectra obtained from thousands of data points collected from thin biological tissue sections as well as inorganic samples. Owing to the MS-based detection principle, IMS has now opened up a new frontier, particularly in the imaging of a variety of small organic molecules, such as endogenous metabolites, and the *in vivo* monitoring of administered drugs (within the animal/human body). The most frequently used ionization techniques are MALDI or SIMS (secondary ion mass spectrometry) (Yang et al. 2010) and recently, the application of NP-assisted laser desorption/ionization (nano-PALDI) has been initiated.

IMAGING MASS SPECTROMETRY (IMS)

Principles of MS and IMS

MS is an analytical technique that measures the mass-to-charge (m/z) ratio of charged atoms, molecules, and molecular clusters/fragments. MS is one of the fastest and most reliable methods for the high-accuracy determination of the mass of analyte particles. A typical mass spectrometer is composed of several functionally distinct components—an ion source, a mass analyzer, and a detector. Within the ion source, the analyte atoms or molecules are ionized by means of various ionization techniques; the analyzer then separates the ions on the basis of their m/z values and the detector outputs electrical signals in response to the reception of separated ions. The mass spectra can then be constructed from these signals (Fig. 1). The development of analyte ionization techniques, including recent progress in the soft-ionization technique, particularly MALDI and electrospray ionization (ESI), has opened up the possibility for the analysis of quite a wide range of molecules using a simple procedure, especially for large biopolymers such as proteins, nucleotides, and polysaccharides. MALDI, in particular, permits the analysis of solid phase samples and has therefore has been adopted in IMS from early studies mainly for biological tissue samples (Stoeckli et al. 2001). The details of this application are further discussed in a subsequent section. The MALDI process is triggered by a laser beam. An organic matrix compound is used to protect the large

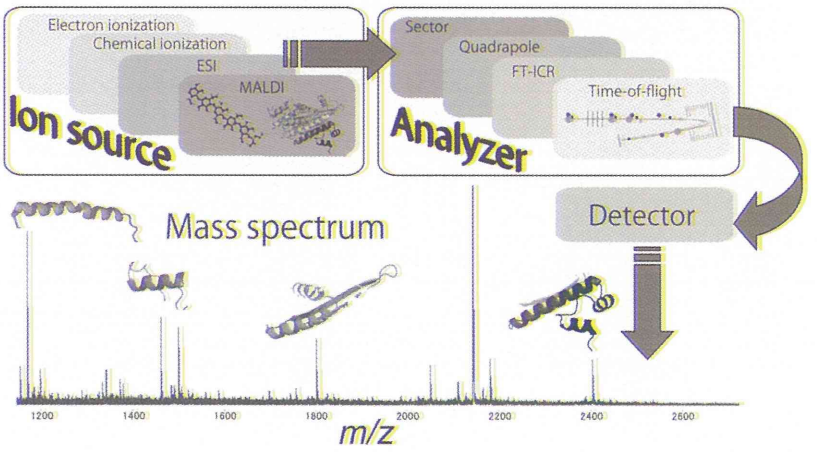

Fig. 1. Principle schematic of mass spectrometer. A typical mass spectrometer consists of three separate components—an ion source, a mass analyzer, and a detector. Numerous variations have been developed for each component based on different principles.

biomolecules from being destroyed by direct contact with the laser beam and to facilitate vaporization and ionization. The currently derived, general versatility of MALDI was established by a momentous effort directed at the development of novel matrix compounds suitable for the ionization of various molecules of interest. This effort has, therefore, facilitated the utilization of MALDI-IMS for the visualization of a variety of molecules (Sugiura and Setou 2010a).

Figure 2 illustrates the general workflow of MALDI-IMS. The basic technique involves the mounting of thin tissue slices on conductive glass slides and application of a suitable MALDI matrix to the tissue section. The slide is then inserted into a mass spectrometer and a focused laser beam is directed at predetermined positions of the tissue slice. The mass spectrometer records the spatial distribution of the molecular species (typically with a 10–200 μm scan pitch). Automated data collection takes 2–6 h, depending on the number of points assayed. Appropriate image processing software is required to import data from the mass spectrometer in order to allow visualization of the ion distribution images and comparison with the histological images of the sample. The unique advantages of MALDI imaging that facilitate the versatility of IMS as a molecular imaging technique are summarized as follows. (1) IMS does not

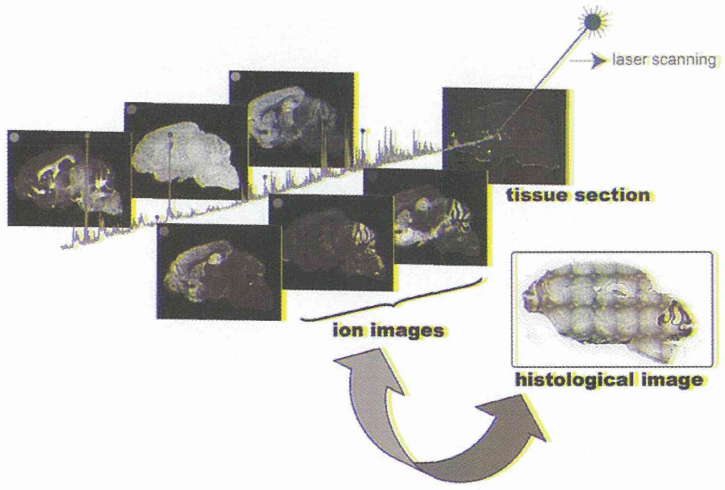

Fig. 2. Schematic representation of matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) procedures. The MALDI laser scans through a set of preselected locations on the tissue (10–200 μm scan pitch), and the mass spectrometer records the spatial distribution of molecular species. Suitable image processing software can be used to import data from the mass spectrometer to allow visualization and comparison with the histological image of the sample.

require any specific chemical labels or probes. (2) IMS is a "non-targeted" imaging method. (3) The simultaneous imaging of multiple types of molecular species is possible. With the unique and powerful detection principle facilitated by MS, the MALDI-imaging mass spectrometry (IMS) can be used for the visualization of the distribution of a large number of biomolecules in cells and tissues, ranging from small metabolite molecules (Khatib-Shahidi et al. 2006) to much larger proteins (Chaurand et al. 2006).

IMS Applications to Areas of Health and Diseases

Current MALDI-IMS applications can be subdivided into two major categories, namely, IMS analysis of large proteins/peptides and IMS analysis of small organic molecules. In the early days, most of the reports on MALDI-IMS were geared at the detection and imaging of proteins or peptides. This area of application particularly targeted the detection of biomarker proteins, which localize specifically in lesions, by utilizing the capacity of IMS for the direct and simultaneous detection of multiple proteins in the tissues. Figure 3a shows one of the earliest medical application studies, which reports the detection and imaging of cancer-specific proteins in a mouse glioma model (Chaurand et al. 2004). As discussed previously, one of the significant advantages of IMS is that a number of cancer/normal specific protein distribution images can be acquired in a non-targeted manner from a single measurement. In addition, disease diagnosis by distinguishing between normal and cancerous biopsy specimens has been attempted by statistical evaluation of such multiple protein expression levels. Figure 3b shows that by applying hierarchical clustering analyses of IMS datasets obtained from human lung specimens, Yanagisawa et al. (2003) achieved successful classification of not only normal and cancerous biopsy samples but also of different cancer types, i.e., primary and non-primary non-small cell lung cancer. Such IMS-based molecular diagnosis studies continue to attract growing attention.

Another major medical application of IMS to the analysis of small molecules is conducted in the pharmaceutical field, e.g., for pharmacokinetic monitoring, pharmacotoxicology, and pharmacometabolomics (Fig. 4). For example, an important phase of drug discovery is determining how a drug-candidate compound is distributed and metabolized within the body. The application of IMS to the monitoring of drug delivery has also attracted much interest. Compared to traditional whole-body autoradiography (WBA) using radio labeled compounds, IMS offers many advantages in the determination of drug distribution. First, IMS allows for the simultaneous and discriminate monitoring not only of the intact drug molecules, but also of their metabolites (Khatib-Shahidi et al. 2006), whereas WBA cannot

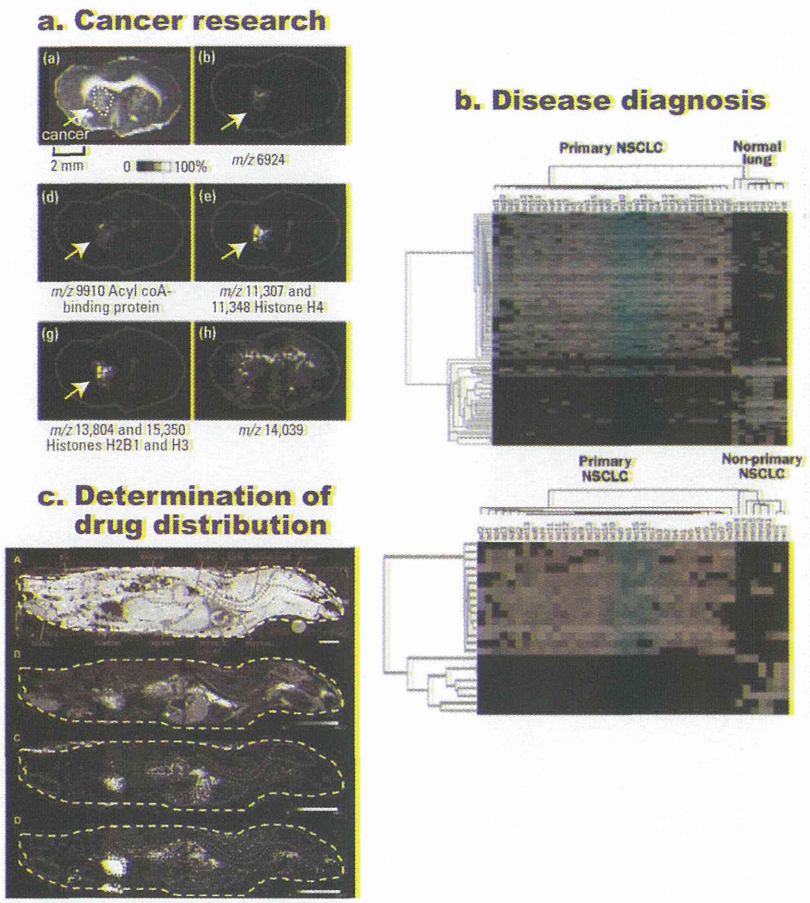

Fig. 3. Representative imaging mass spectrometry (IMS) applications in areas of health and diseases. Shown are representative examples of IMS application to brain cancer research using the mouse glioma model (a); human disease diagnosis of healthy and different types of lung cancers (b); and determination of pharmacokinetics in the whole animal body (c). NSCLC = non-small cell lung cancer. Reprinted from Chaurand et al. 2004 (a); Yanagisawa et al. 2003 (b); and Khatib-Shahidi et al. 2006 (c) with permission of ACS Publications (a and b) and Elsevier, Ltd. (c).

distinguish these molecules. Thus, IMS can be used to determine whether or not medicinally intact drugs have reached the target organs. Secondly, IMS can be used to visualize the distribution of drugs at a lower cost and in a much shorter time than with detection using isotopes. Figure 3c shows the detection of drugs that have been delivered orally to mice. In this study, the distribution of the antipsychotic drug olanzapine and its metabolites in

the sagittal section of an intact (whole) rat was successfully investigated 6 h after administration. This study clearly showed the distinct distribution of intact drugs and their metabolites; the intact drug reached the target organ (the brain), whereas its metabolites were localized in the bladder.

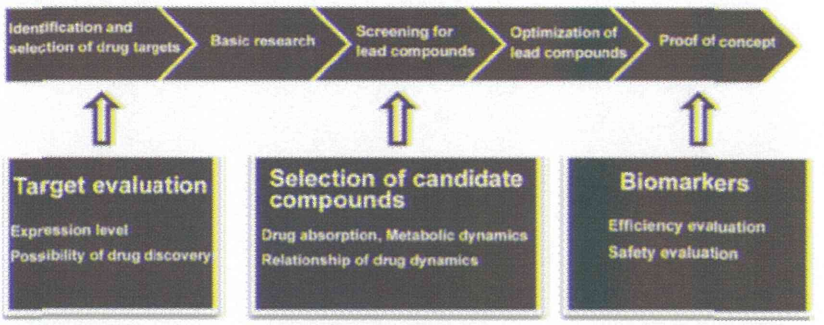

Fig. 4. Processes of new drug development and areas of application of imaging mass spectrometry (IMS) in each step. New drug development can be divided into several processes. IMS can be widely applied to many of the steps along the way through the ability to visualize various molecules.

APPLICATION OF NANOPARTICLES TO IMS

Utilization of a Variety of NPs in Basic MS Research

As described previously, MALDI had its genesis as a soft-ionization technique employing NPs (Tanaka et al. 1988). Since then, rapid technical progress over the ensuing couple of decades has made NP handling much easier, and studies on the utilization of NPs as ionization assisting reagents have been intensively reported. The main motivation behind the use of NP-assisted ionization is to overcome the limitations of MALDI, particularly for the detection of molecules in the low mass range, without interference from matrix-derived ions. The low m/z region of a MALDI spectrum contains a large population of ions from biological metabolites as well as matrix-related adduct clusters and fragments, which are dominantly observed in the MALDI mass spectrum. This high density of ions increases the risk of sharing of the same mass window by matrix ions and analyte molecules. The utilization of NPs as a matrix is one of the effective methods for prevention of this problem, because the NPs produce few background ions. Figure 5 shows an example in which reserpine, an indole alkaloid antipsychotic and antihypertensive drug, was analyzed using both fNP and α-cyano-4-hydroxycinnamic acid (CHCA) as respective matrices. The spectra presented clearly demonstrate the complete elimination of background ions by use of NPs (Sahashi et al. 2010). Furthermore, the

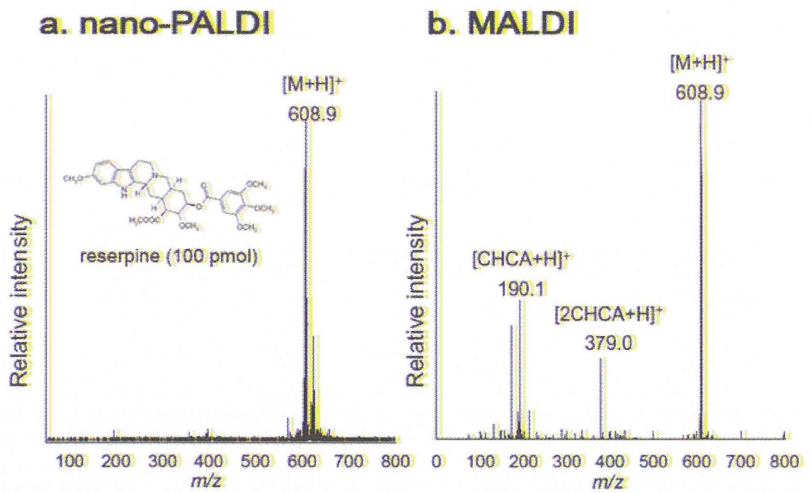

Fig. 5. Comparison between mass spectra obtained by nanoparticle-assisted laser desorption/ionization (nano-PALDI) and matrix-assisted laser desorption/ionization (MALDI). Mass spectra of reserpine, an indole alkaloid antipsychotic and antihypertensive drug, obtained by both nano-PALDI and MALDI-mass spectrometry in which functional nanoparticle (fNP) and α-cyano-4-hydroxycinnamic acid (CHCA) were used as matrices, respectively. This example clearly demonstrates the complete elimination of background ions by use of NPs. Reprinted from Sahashi et al. 2010 with the permission of Elsevier, Ltd.

use of NPs has improved the homogeneity of analyte distribution in the samples on the target plate, which could increase sample-to-sample reproducibility. In comparison, the crystallization process in MALDI inevitably causes artificial localization of analyte molecules within the sample; therefore, researchers have had to locate a *hot spot* where the analytes were concentrated by moving the laser irradiation spots. This is a time-consuming process and one of the major reasons for the lowered quantitative performance of MALDI.

In addition to these general advantages of NP-assisted ionization, a variety of characteristic NPs have been used for analyte-specific MS research (as summarized in Table 1). For example, graphite NPs have been utilized for the detection of relatively large molecules such as peptides and proteins from liquid solutions, with sensitivities in the pico- to nanomole range (Sunner et al. 1995). Bifunctional magnetic iron oxide particles immobilized on silane have also been used for the analysis of proteins and peptides (Chen and Chen 2006). Recently, citrate capped gold nanoparticles (AuNPs) have been utilized for the analyses of important signaling biomolecules—progesterone, testosterone, and cortisol (Wu et al. 2009), which are difficult to detect using conventional organic matrices. Silver

Table 1. Representative nanoparticle assisted laser desorption/ionization technique and its applications. Listed are representative mass spectrometry studies in which various nanoparticles were utilized as ionization enhancing reagents; applied to the measurement of biological molecules.

Name of NP	Core metal	Diameter	Analyte	Reference	Imaging
AgNPs	Ag	3.84±0.45nm	Fatty acids	Hayasaka et al. 2010	yes
		34±3nm	Estrogen	Chiu et al. 2008	yes
					yes
MnNPs	MnO_2, $Mn2O_3$	5.4±0.2nm	Ginsenosides	Sahashi et al. 2010	yes
fNP	Fe_2O_3	3.7nm	Phospholipids	Moritake et al. 2009	yes
			Sulfatide	Ageta et al. 2009	yes
			Lipids, peptides	Taira et al. 2008	yes
AuNPs	Au	4.3±0.7nm	Glycosphingolipids	Goto-Inoue et al. 2010	yes
		13.2±1.2nm	Progesterone, Cortisol, Testesteron	Wu et al. 2009	no
TiO_2 NPs	TiO_2	‹0.05μm	Trypsinogen	Watanabe et al. 2009	no

Ageta et al. 2009. Med. Mol. Morphol. 42: 16–23.
Chiu et al. 2008. J. Am. Soc. Mass. Spectrom. 19: 1343–1346.
Goto-Inoue et al. 2010. J. Am. Soc. Mass. Spectrom. 21: 1940–1943.
Hayasaka et al. 2010. J. Am. Soc. Mass. Spectrom. 21: 1446–1454.
Moritake et al. 2009. J. Nanosci. Nanotechnol. 9: 169–176.
Sahashi et al. 2010. Food Chem. 123: 865–871.
Taira, S. et al. 2008. Anal. Chem. 80: 4761–4766.
Watanabe et al. 2009. J. Mass Spectrom. 44: 1443–1451.
Wu et al. 2009. J. Am. Soc. Mass. Spectrom. 20: 875–882.

nanoparticles (AgNPs) capped with several types of functional groups have been used for the detection of sulfur drugs and biothiols (Shrivas and Wu 2008). It has also been demonstrated that AgNPs can selectively ionize cholesterol, phosphatidylcholine, and carotenoids (Sherrod et al. 2008). Moreover, AgNPs have also been used for the determination of small molecular hormones, such as estrone, estradiol, and estriol (Chiu et al. 2008). In another study, titanium dioxide (TiO_2) NPs modified with urea have been shown to increase the ionization efficiency of analytes owing to the photocatalytic effect of TiO_2, which was easily activated by UV irradiation. Furthermore, the modified TiO_2 NPs could also be applied to the detection of large proteins of sizes greater than 20 kDa, such as trypsinogen (Watanabe et al. 2009).

Nano-PALDI for IMS

The usefulness of MALDI-based IMS was briefly reviewed in the preceding sections; however, utilization of the NP-assisted ionization technique is an attractive alternative to MALDI. In this context, utilization of NPs in IMS is expected to be a quite useful tool, especially for the imaging of small molecule distribution, and therefore, this state-of-the-art imaging technology, nano-PALDI-based IMS, and its future perspectives are discussed in the following sections.

Current Limitations of MALDI-IMS and Nano-PALDI-IMS as a Solution

Despite the promising capability of MALDI-IMS, this technique still has several critical limitations. An important challenge is the improvement in spatial resolution toward ion imaging within cellular organelles, which requires resolution at the sub-micrometer level. However, when MALDI is employed as an ionization technique for IMS, the nature of the MALDI process requires the formation of analyte-matrix co-crystals on the tissue section. The typical size of these co-crystals is >50 μm; they function to protect the analyte molecules from direct laser irradiation, i.e., act as a "cushion" and eventually enhance the soft ionization of biomolecules. Unfortunately, this crystal size effectively limits the spatial resolution of IMS to as large as the crystal size. In this regard, imaging with SIMS, a matrix-free ionization technique, has already achieved submicron spatial resolution. In SIMS, the use of a tightly focused ion beam for ionization offers a resolution at several tens of nanometers and has been successfully used to visualize sub-cellular structures in biological samples (Monroe et al. 2005; Ostrowski et al. 2004). However, SIMS is a much "harder" ionization method than MALDI, and consequently, it is not the best choice for intact ionization of various biomolecules because heavier molecules (<1000 Da) and molecules with easily fragmented groups cannot be ionized in their intact from using SIMS (Kraft et al. 2006). In order to overcome these issues, the current authors have reported an NP-assisted laser desorption/ionization (nano-PALDI)-based IMS technique, in which the organic matrix is replaced with NPs, and therefore, the matrix crystallization process is eliminated (Taira et al. 2008). Figure 6 presents a simple illustration of the relationship among MALDI, SIMS, and nano-PALDI.

This novel nano-PALDI-IMS technique affords high-resolution imaging of complex biological specimens (Taira et al. 2008). For this purpose, functional nanoparticles (fNPs) with a diameter of 3.7 ± 0.1 nm were also developed. The inset of Fig. 7 shows the structure of the developed fNPs. Surface-positioned silicon dioxide (SiO_2) groups could be used for attaching various chemical groups, and in the study, hydroxyl