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Why don't biologists use SIMS? A critical evaluation of imaging MS

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Abstract

Secondary ion mass spectrometry is commonly used to study many different types of complex surfaces. Yet, compared with MALDI and ESI–MS, SIMS has not made a significant impact in biological or biomedical research. The key features of the technique, namely high spatial resolution, high detection efficiency of ions spanning a wide *m/z* range, surface sensitivity and the high scan rates seem to match ideally with several questions posed at the cellular level. To this date, SIMS has had only limited success in the biological arena. Why is this and what is needed to change this? This discussion paper will critically review the advances and the usefulness of SIMS in biomedical research and compare it to other approaches that offer spatially resolved molecular information available to a researcher with a biological interest. We will demonstrate that the type of information generated by the various incarnations of SIMS is strongly dependent on sample preparation and surface condition and these phenomena are only poorly understood. Modern approaches such as the cluster gun developments, ME-SIMS, gold coating and MALDI stigmatic imaging on a SIMS instrument might change the perception of SIMS being a tool for semiconductor manufacturers and physicists, and might persuade biologists to use these innovative mass spectrometric imaging tools.

Keywords: ME-SIMS; MALDI; Imaging mass spectrometry; Time-of-flight mass spectrometry; Biomolecules

1. Introduction

At present there is a growing interest in the examination of the spatial organization of biomolecules directly from biological tissue. Molecular biology thrives on molecular imaging techniques that aim at the investigation of the relation between spatial organization, structure and function of molecules in biological systems. Through the use of multiple fluorescent labels it is now possible to study the spatio-temporal behavior of selected biomolecules in parallel in a single experiment [1]. Such microscopy studies have already been used to substantiate the intuition that a protein's function is correlated with its localization in the cell [2,3] and to demonstrate that several diseases are associated with altered molecular distributions [4–6]. Imaging mass spectrometry is one of the few techniques that have the potential to overcome the requirements for fluorescent labels. The recent advances in mass spectrometry now enable us to investigate the dynamics of large molecular assemblies, even follow chaperone assisted molecular folding [7]. The sensitivity and mass range of many modern mass spectrometers is no longer limiting for groundbreaking biological studies. Throughout the last five decades there have been many different incarnations of imaging mass spectrometry. In contrast to the non-destructive spectroscopic techniques, MS imaging relies on the removal of extremely small amounts of molecules from the surface under study. For this purpose routine methods for desorption and ionization can be used that are commonly employed in mass spectrometric investigations. Spatial resolution, molecular selectivity, depth of analysis, sensitivity, destructiveness and the required sample modification are key issues in molecular imaging of biological surfaces. No technique exists (yet) that does not compromise on at least one of these issues.

Like no other mass spectrometric technique, secondary ion mass spectrometry (SIMS) has a long history in high-resolution visualization of elements and organic molecules at surfaces.

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Yet, in biomedical research SIMS is seldom used. In this discussion paper we will try to identify why this is and what potential avenues exist that will advance the field into the biomedical arena. Some statements will be bold to start a discussion on this topic and we realize that many subtleties exist; yet these perceptions exits and need to be addressed. First, a thorough investigation of the questions posed by biologists/biochemist is called for before we go into the merits of imaging mass spectrometry and SIMS in particular. Here we will list a number of general questions we are often confronted with and in the following sections discuss them in the light of imaging MS methodologies.

1. What type of biomolecules can be studied with imaging MS?
2. What is the detection limit of these molecules in cells and tissue?
3. What is the spatial resolution that can be obtained?
4. Can in vivo studies be performed?
5. Can small modifications or alterations (such as post-translational modification on proteins) be detected?

Admittedly, although these questions are of a general nature there is no general answer to any one of them. The answer often depends on a number of different factors. In the following sections some of these factors will be highlighted and discussed for the examination of cells and tissue. We will specifically address issues related to the study of intact molecules, although in some cases relevant and informative fragmentation processes will be discussed.

2. Biomolecular ionization techniques for imaging MS

The molecular classes that can be studied with imaging mass spectrometry strongly depend on the ionization technique/methodology used and the environment from which the molecule needs to be liberated from. Historically, imaging MS started to look at elemental ions. In particular the developments in dynamic SIMS on biological systems demonstrated that using high-resolution primary ion beams beautiful images could be generated that visualize the presence of elements like calcium, sodium and potassium in different organelles in the cell. In some cases where certain pharmaceuticals contain characteristic elements the distribution of these drug molecules can be imaged by investigating the elemental fragmentation products. Boron neutron capture therapy of tumors, where SIMS was used to show accumulation of the pharmaceuticals in cancer cells, represents arguably the most successful biological SIMS application [8,9]. Yet for the biochemist the elemental distributions do not answer the most relevant questions. Biomolecules like lipids, peptides and proteins are far more interesting in his eyes. SIMS can be used to generate characteristic fragment ions of these types of molecules when they are applied as standards onto an inert substrate. Abundant lipids such as PC and PE, PA and to a lesser extent ceramides and PI's have been desorbed and ionized with SIMS [10], but the less abundant phospholipids such as PIP's and cardiolipins that are much more interesting from a biological point of view are not (yet) observed. Two general approaches can be distinguished to extend the applicability of SIMS to biomolecular imaging; primary ion beam modifications and surface modifications. Both aim to modify the way the energy of the primary ions is dissipated in the surface in order to increase the survival yield and ionization efficiency of intact biomolecular species. It still remains to be seen if the improvements promised by these techniques can advance the SIMS field to be competitive with MALDI imaging MS in such a manner that it will attract a similar scale of interest from biologists.

2.1. Matrix enhanced SIMS (ME-SIMS)

ME-SIMS [11] can be considered a combination of MALDI and SIMS. The application of an organic acid to a surface dramatically enhances the ionization efficiency of larger molecules. In an extensive study Wu and Odom demonstrated that peptides, proteins and small oligonucleotides could be analyzed with a variety of matrices at sub-picomolar sensitivities. In addition, this technique has been shown to be applicable to the analysis of synthetic polymers [12,13], and recently has been applied to the direct analysis of tissue [14,15]. For these purposes common MALDI matrices are used. The sensitivity and obtainable spatial resolution strongly depend on the combination of sample type, sample preparation protocol and the matrix used. The analysis of standards and pure compounds is readily facilitated by ME-SIMS and generally spectra very similar to MALDI spectra are obtained. Fig. 1 shows a comparison for three different classes of compounds in the same mass range taken on a TRIFT-II ToF-MS. The spectra of polyalanine in frames 1E and 1F show no fragmentation whatsoever yielding a small advantage over metal coating these materials [16]. It is obvious that the results are spectrally very comparable. Yet there are a few differences to consider.

The ionization mechanisms are outwardly very different. These ME-SIMS experiments used a 12 kV In^+ beam rastered

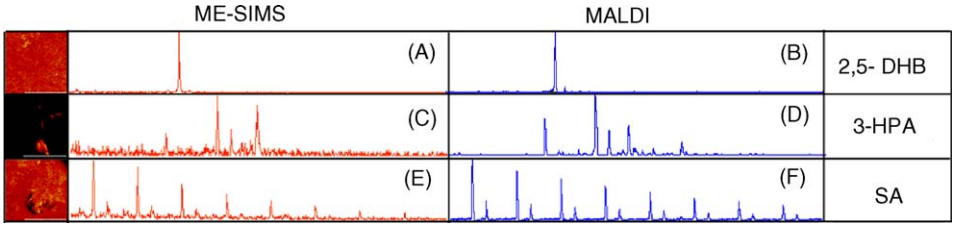

Fig. 1. A comparison between MALDI and ME-SIMS of three different molecular classes. (A and B) are the positive ion spectra of the oligosaccharide maltoheptaose in 2,5-dihydroxybenzoic acid; (C and D) are the small oligonucleotide tetrathyrosin (T4) in 3-hydrocypicolinnic acid taken in negative mode; (E and F) are the positive ion spectra of a synthetic peptide polyalanine (AlA)n in Sinapinnic acid.

over a 150 μm × 150 μm surface. The MALDI results were acquired from a 200 μm diameter laser spot using 337 nm light from a N_2 laser while extensively moving the sample surface. The static SIMS conditions ensure that less than 1% of the surface area is sampled, i.e. 100 μm^2, whereas in MALDI the irradiation area was several orders of magnitude larger. The primary ion beam in the SIMS case interacts exclusively with the topmost layers of the surface. In the MALDI case the ablation crater is significantly deeper and is estimated to be 1 μm or more, depending on laser fluence. In order to obtain these similar spectra the interaction volume during MALDI is estimated to be five orders of magnitude larger, which would indicate that ME-SIMS is far more sensitive than MALDI. (In this comparison the MALDI data were acquired with an ADC while the ME-SIMS data were acquired with a TDC) The images in Fig. 1 also reveal that for ME-SIMS the actual signal can originate from specific areas, or hot-spots [17]. A conventional MALDI experiment of the same area would deliver one single spectrum and would not be able to reveal hot-spots within the laser focus. It seems that ME-SIMS has only advantages to offer over MALDI from the ionization efficiency and the information quality point of view so this cannot be the reason why biologists do not use SIMS and related techniques. The reason ME-SIMS has not been used is the molecular weight range. For larger biomolecules (>5 kDa) the signal-to noise ratio of ME-SIMS drops dramatically while with MALDI a good sensitivity can still be obtained. Although intact protein ions up to 17 kDa have been observed with ME-SIMS these spectra had to be obtained with increased primary ion dose and took significantly longer to acquire than the MALDI data. Moreover, ME-SIMS has only been demonstrated for extremely large protein concentrations that were purified prior to sample preparation. Given the current state of the technique it does not seem possible to liberate, ionize and detect intact, low abundance proteins with SIMS or ME-SIMS.

The discussion in the previous paragraph demonstrates that ME-SIMS works well for standards and ''artificial'' samples. Sample preparation plays a decisive role for direct ME-SIMS imaging of tissue. Several considerations determine the choice of matrix and deposition method for direct tissue imaging. In order to optimally benefit from the high-resolution of the primary ion beams used for ME-SIMS, the matrix crystals need to be smaller than the features of interest. If cellular features are targeted, crystals typically need to be smaller than 500 nm. Others and we have observed that smaller crystals typically lead to lower sensitivity for the detection of intact biomolecules. In order to form small crystals and limit diffusion of analytes, the application of the matrix needs to occur through small droplets arriving at the surface. Sufficient time needs to be provided to solvent extract the biomolecules of interest from the tissue surface into the droplet prior to crystal formation, yet slow solvent evaporation leads to bigger crystals. Different matrix application procedures have been experimented with ranging from electrospray deposition (smallest crystals), pneumatically assisted spray deposition (TLC sprayer), picoliter droplet spotting and dried droplet preparation (largest crystals).

The crystallization process is heavily influenced by the presence of salts and surface-active compounds. They can disrupt the matrix crystallization process, thereby hindering efficient ion generation, complex with the analytes and matrix molecules, and compete with the limited charge available (typically <0.1% of the total material ablated). Arguably the most significant limiting factor for biological ME-SIMS analyses concerns the surface sensitivity of the SIMS technique: surface-active compounds are enriched in the surface of the matrix crystals. Consequently ME-SIMS experiments of tissue or cells frequently yield lipid-like ions. Despite these limitations, ME-SIMS has not enjoyed as much attention as perhaps it should have in the SIMS community. Presumably this is because of its lack of surface specificity (in SIMS the ions are obtained from the top few monolayers whereas in ME-SIMS the origin of the ions is defined by the matrix application step) and because of the negative connotation associated with the term ''matrix'' in this field.

2.2. Surface metal coatings

Metallization of (organic) samples with silver and gold has been shown to increase secondary ion yields of intact molecular ions in SIMS [18–23], this technique has been named metal-assisted (MetA) SIMS. It provides increased sensitivities for large analytes as diverse as polymers, dyes, fatty acids, lipids and peptides. In addition, the thin metal layer provides a conductive contact thus effectively converting insulating samples into conducting samples, thereby removing the need for charge compensation. The mechanism behind this increased sensitivity has been attributed to analyte migration onto the nano-islands produced by the deposition and to the catalytic properties of these nano-islands. Also increased stopping power and ''self-sputtering'' [24,25] could be potential causes for the enhanced sensitivity. A complication of using sample metallization is that the ions observed are frequently adducts with the metal and metal clusters. This makes peak assignment more difficult, especially for samples whose components are unknown prior to the experiment.

SIMS of naked tissue sections and single cells is frequently dominated by compounds associated with membranes: phospholipids, lipid fragments and cholesterol. Nygren et al. have reported increased sensitivity for cholesterol from rat kidney tissue sections using thin silver coatings [21]. Thin metal coatings (gold, silver and platinum) are easily and quickly deposited, with full control of the thickness, using commercial sputter coaters (evaporative coaters produce a lot of heat that can damage fragile samples). Yet again, this surface modification seems limited to lower molecular weight biological compounds (≪5 kDa). In our hands, the application of a thin metal coating to rat brain tissue greatly enhanced the sensitivity for biomolecular species upto 3 kDa [26]. A good measure of proper layer thickness is the appearance of gold cluster ions in the mass spectrum taken from the tissue. When the metal coating becomes too thick, it will obscure the biological layer underneath. This typically happens for coatings exceeding 5 nm. Recent reports have demonstrated that a thin metal coating also significantly improves MALDI analyses, both in imaging and conventional MALDI applications [27].

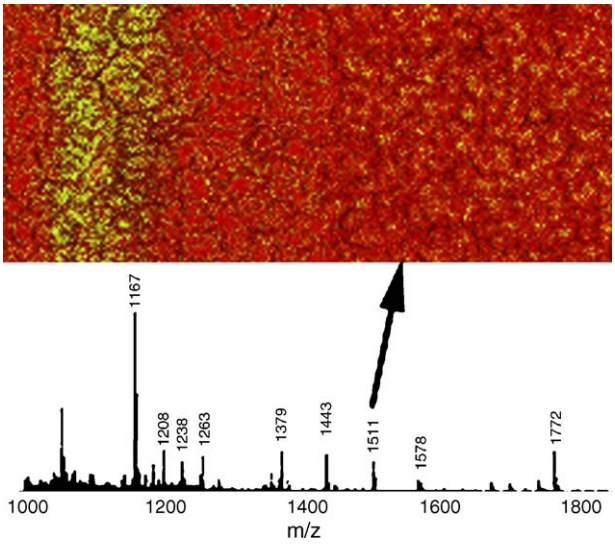

Fig. 2. Meta-SIMS of a gold-coated tissue section of the third ventricle region of a rat brain with the corresponding spectrum.

Fig. 2 shows a result of SIMS imaging of gold-coated rat brain tissue. A 1 nm thick layer of gold was sputter coated on the sample surface using a Quorum Technologies (Newhaven, East Sussex, U.K.) SC7640 Sputter Coater. Contrary to an uncoated surface a very rich spectral signature is observed between 500 and 2500 *m/z*. Many of the peaks revealed specific localization on the surface of individual cells or regions of the brain. In the image in Fig. 2 the total ion image in red is overlaid with the image of the *m/z* 1511 peak in green. To date this peak has not been identified. It shows an increased intensity in the region of the central ventricle and is present on the surface of the majority of the neuronal cells. The fact that this peak is not yet identified points to another drawback of ToF-SIMS imaging. Most commercial SIMS instruments lack tandem MS capabilities that are a *sine qua non* for identification purposes with mass spectrometry. As a result it is possible to make beautiful pictures such as presented in Fig. 2, but in order to understand the meaning and relevance of new peaks found with such complex samples we have to refer to conventional tandem MS approaches. This identification is complicated by the fact that when applying more routine MS identification strategies on tissue homogenates typically another ionization technique is employed that might not generate the same ions.

As the amenable mass range of SIMS experiments increases, tandem mass spectrometry facilities will undoubtedly be implemented. However, at present they are lacking.

The workflow depicted in Fig. 3 describes a general approach to reveal molecular identity and spatial organization

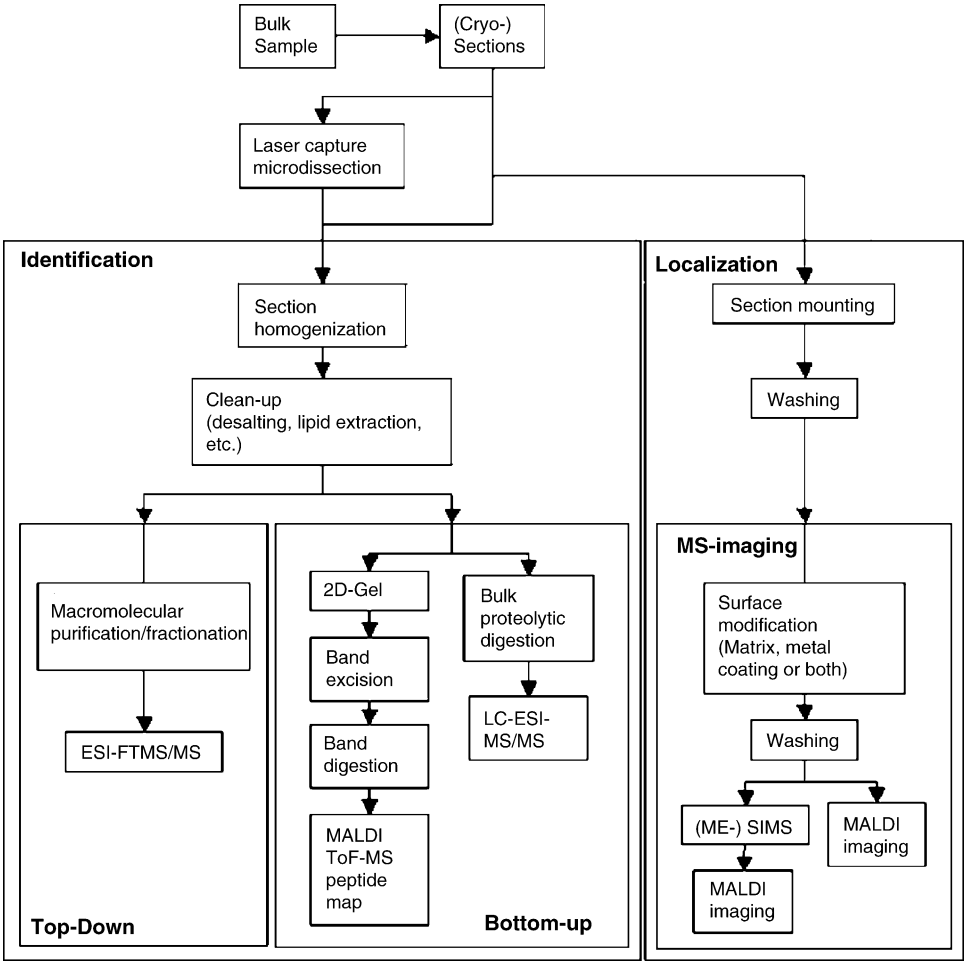

Fig. 3. Workflows for MS localization and identification of proteins from tissue sections.

for unknown proteins and peptides. Of particular interest is the application of laser capture micro dissection (LCM) where selected individual cells or groups of cells can be isolated for further analysis. This is a powerful alternative for low resolution imaging as standard sensitive proteomics approaches [28] can be employed (but will be very slow). The different identification strategies sketched in Fig. 3 could provide molecular structural details such as amino acid composition and sequence, various labile post-translational modifications and could even be made quantitative. All these features still lie far beyond the capabilities of imaging MS. The absence of a connection in Fig. 3 between identification and localization is unfortunate. As a result, the different approaches all need to be carried out in concert to provide the broadest insight into the cohort of proteins and peptides in tissue. The tables of proteins and peptides originating from the identification strategy provide a road map for the interpretation for the spectral peaks found in imaging MS. The big gap between the left and the right side of Fig. 3 is a major limitation for biological SIMS imaging.

2.3. Matrix assisted laser desorption and ionization (MALDI) microprobe imaging

Many different research groups have early on speculated about the possibilities of visualizing macromolecular distributions directly from organic surfaces. Sofar, MALDI–MS imaging is the only technique that generated bio-macromolecular images from biological samples directly [29–32]. To date no examples exist where unknown proteins have been localized and identified from a complex biological surface using any of the SIMS related techniques. MALDI based imaging techniques on the contrary have shown examples of such applications [33]. A major advantage of MALDI over SIMS based techniques is its ability to generate intact macromolecular ions. The spatial resolution that has been obtained in various MALDI imaging application directly on tissue is determined by the spot size that can be generated on the surface of interest. The spot sizes used in MALDI imaging MS are not limited by the nature of the light used, but rather by the configuration of the source region of the mass spectrometer, which is not optimized for creating a tightly focused microprobe. This requires the last (focusing) objective to be placed inside the vacuum near the sample plate, blocking the desorbed ions or influencing the accelerating field. Recently, Spengler and Hubert circumvented this with a clever design in which the last focusing lens contains a central hole fitted with a steel tube through which ions can be extracted [34]. In this way a microprobe of approximately 1 μm was achieved. Nevertheless, until now no high-mass ($m/z > 5000$) protein signals have been recorded from spot sizes of 1 μm or smaller. Ens et al. have recently demonstrated high-mass sensitivity with a 5 μm spot size on dried droplet prepared samples up to 100 kDa on a qQ-ToFMS [35]. In a publication by Dreisewerd et al. it was observed that the laser threshold fluence (J/m^2) for the generation of protein ions from matrix crystals increases rapidly for decreasing spot sizes [36]. This higher fluence causes extensive fragmentation, thereby limiting the sensitivity and the specificity.

Yet, as an ionization technique, MALDI has revolutionized the investigation of biomolecules. It is sensitive enough to investigate low level proteins in the bottom up approach as illustrated in Fig. 3. Mass spectrometric microprobe imaging with MALDI can be achieved on commercial ''standard'' ToF instruments. A smart piece of software that controls the sample stage and the data collection timing is the only additional instrumental feature needed to perform MS imaging experiments, with the limitations discussed above however. The capability to perform automated tandem MS experiments is a tremendous advantage of the implementation of MS imaging on commercial instruments with data dependent experimentation. It combines identification strategies with localization.

As the international biology community has accepted MALDI-ToF for many experimental purposes it has been easier to accept this as a sensitive molecular imaging technique. The principal difference between MALDI and SIMS is that MALDI has been providing the chemical information that is desired; with SIMS this is the exception rather than the rule.

2.4. Cluster SIMS

The development of liquid metal ion guns as a primary ion source for SIMS experiments in the 1990s revolutionized SIMS imaging in that it leads to high spatial resolution imaging mass spectrometry becoming routine. Today many high-resolution imaging mass spectrometers use either liquid metal ion guns or technologies arising from liquid metal ion guns (bright point sources). The latest ion guns use polyatomic primary ions such as gold clusters [37–40], bismuth clusters and Buckminster fullerene [41,42]. Enhanced sputtering by polyatomic primary ions has been known for more than four decades: in 1960 the groups of Kistemaker and co-workers [43] and Grønlund and Moore [44] independently demonstrated that polyatomic projectiles sputter more material than that expected from independent atomic projectiles. In the late 1980s during the development of the SF_6^- ion source, again reporting significantly enhanced sensitivities, Appelhans and Delmore were the first to show that enhanced yields are produced with a concomitant increase in surface damage, but that the yield enhancement surpasses that of the damage [45]. The new developments in polyatomic primary ions have been making these sensitivity enhancements compatible with imaging mass spectrometry by designing the columns, and the ion source, such that they can provide the focused ion beam necessary for high-resolution microprobe imaging. These are the gold [46] and bismuth cluster guns, and the C_{60}^+ gun developed by Vickerman and workers [41,42]. New gold (bismuth) primary ion guns combines high-mass resolution capabilities, <700 ps pulse width, and high spatial resolution capabilities. Examples of images obtained from tissue sections of a mouse brain using a focused Au_3^+ primary beam have been shown [40]. The images of cholesterol, stearic acid (ST), phosphatidylinisitol (PI) and other high-mass peaks clearly demonstrate the benefit of polyatomic primary ion SIMS for imaging of biological samples. The spatial resolution of the C_{60}^+ is worse, spot size 2–3 μm [42], but it offers higher yields than Au_3^+: a direct

comparison revealed C_{60}^+ gave significantly more signal, factor 2–3 [41]. Molecule specific imaging of combinatorial libraries and parallel mass analysis of multiple beads, clearly demonstrated the utility of higher sensitivity SIMS for imaging mass spectrometry [47]: the spatial analysis allows the analyst to rapidly determine if molecular ions are correlated (from the same bead), and the higher sensitivity allowed a family of small peptides to be identified and sequenced.

Several massive polyatomic primary ion systems have been developed, exhibiting extreme examples of the benefits of polyatomic primary ions. Massive glycerol cluster ions, masses extending to 10^6 to 10^7, have been used to generate intact molecular ions of peptides [48–51]. McMahon et al. have shown how the large sputter yields of these massive cluster ions can remove the damage accumulated during atomic ion SIMS, thereby allowing ''organic ion imaging beyond the limit of static secondary ion mass spectrometry [50].'' However, this ion source is now seldom used: it produces a high-energy beam of ions and a high-energy beam of neutrals (presumably due to dissociation of larger ions), both of which contain ions/neutrals covering a wide mass range and with a wide range of energies. Consequently, it cannot be focused to small spots and cannot be pulsed with high time resolution, making it incompatible with linear extraction time-of-flight instruments. Nevertheless, it was presumably the development of ESI, MALDI and liquid metal ion guns that was responsible for the present disinterest in massive glycerol cluster ionization: if it provided information unavailable by other means these difficulties would have been addressed. For example, Aksyonov and Williams have demonstrated that good quality mass spectra can be obtained using this ion source (this time the analyte ions were produced by impact of the analyte doped glycerol clusters) with an *orthogonal* time-of-flight mass analyzer [52].

Similarly Tempez et al. have used an orthogonal time-of-flight mass analyzer to obtain high-mass resolution SIMS spectra using massive cluster ions, clusters with masses >80,000 Da [38]. Using the massive Au_{400}^{4+} cluster primary ion they demonstrated ion yield enhancements of 1000, and signal-to-noise enhancements of 20 for the molecular ions of various peptides when compared to the atomic primary ion Au^+. Moreover, this massive cluster ion was shown to produce minimal surface damage. The low energy per nucleon of the massive cluster ion, 100 eV for the experimental conditions reported, limits the penetration depth of each nucleon to the upper surface region, and the enhanced sputtering of the large, multiply charged cluster ion efficiently removes any damaged material. Consequently, minimal damage cross-sections were observed. These massive cluster ions demonstrated superior analytical performance to small gold clusters such as Au_5^+: The molecular ion yields were found to initially increase with ion dose before reaching a plateau value (intense ion signals were observed after the static SIMS limit), whereas the ion yields produced by Au_5^+ began to decrease immediately, and were reduced by a factor of 10 before the static SIMS limit was reached [38]. This ion source has not been used for imaging experiments because of the large spot size, of approximately 7 mm^2. Nevertheless, remarkably it has subsequently been shown that laser irradiation of tissues after massive gold cluster ion impact can produce intact molecular ions of lipids and proteins [53,54]. After implantation of 5×10^{12} Au_{400} clusters per square centimeter in a section of a brain of Sprague–Dawley rat, laser irradiation produced protein peaks up to 30 kDa, one of which was identified as histone H4. This offers an alternative method to analyze the large molecules of a sample without employing MALDI matrices and would permit images to be obtained through using either a highly focused laser beam or the ion optical microscope [55]. Finally, Schenkel et al. have demonstrated that significant improvements in secondary ion yields can be obtained using highly charged atomic ions like Au^{69+} the Xe^{44+} [56]. However, such primary ions are presently beyond the reach of most analytical laboratories.

2.5. DESI

A new and perhaps promising technique for macromolecular imaging that has recently emerged from the lab of Cooks and co-workers is desorption electrospray ionization (DESI) [57]. An atmospheric ionization technique that aims an electrospray generated jet at a surface in front of the inlet of a mass spectrometer. Highly charged liquid droplets entrained in a gas jet collide with a surface where, upon impact, they release intact macromolecular ions from many different surfaces. It has been show that large areas can be examined and the first imaging experiments have already been attempted on TLC plates [58] and tissue [59]. Although there are still damage, sensitivity and resolution issues to address, the technique holds the promise of soft, local, liquid atmospheric desorption and ionization. This could resolve one of the problems often forwarded by biologists, namely the desire to perform imaging experiments away from the harsh vacuum environment of the mass spectrometer.

3. MALDI stigmatic imaging on a SIMS instrument

There is one area where the physicists from the SIMS community and the biochemists from the mainstream MS community meet, namely the use of the instrumental developments from the SIMS community for MALDI applications. One good example of such synergistic work is the application of stigmatic ion imaging [55,60], a SIMS development, for MALDI examination of complex surfaces [61]. Stigmatic or microscope mode ion imaging is a very elegant ion imaging technique that differs fundamentally from microprobe mode imaging. It uses stigmatic ion optics to transport a magnified secondary ion image from the sample to a 2D-detector. As such, it decouples the obtainable spatial resolution from the spot size of the desorption and the ionization beam. Instead, the spatial resolution is governed by the quality of the ion optics and the spatial resolution of a 2D detector. This offers numerous advantages for imaging of complex surfaces. The principle of position correlated ion detection instead of position correlated ion generation removes the need for a tightly focused microprobe for high lateral resolution imaging MS. The maximum ionization area is limited only by the field-of-view of the mass spectrometer defined by its

stigmatic ion optics. As a result, the resolving power, different from the pixel size of 1 μm, has been show to be as low a 4 μm at normal MALDI laser fluences [61] within a field of view of 200 μm diameter in one single laser shot. The speed of analysis is no longer limited by the large amounts of spots that need to be analyzed in a microprobe mode imaging experiment, and images can be generated at a speed limited by the repetition rate of the laser [15]. Larger areas can be analyzed and high-resolution images can be concatenated to form larger field-of-view (FOV) images. For the TRIFT-II system used in our laboratory, the FOV is approximately 250 μm in diameter. This offers a much greater versatility in the choice of the ionization methodologies than for microprobe mode imaging, and allows for instance high-resolution MS imaging with infrared MALDI [62].

Microscope mode ion images were directly recorded off the phosphor screen using a CCD camera. Triggers for the CCD camera and oscilloscope/PCI-digitizer card are provided by a photodiode, which picks up the light scattered from a beam attenuator. The analysis time of one cycle of a microscope mode experiment is dependent on the selected mass range and takes maximally 900 μs. Within this time a series of ion images, separated in time, arrives at the phosphor screen. For each laser shot, an ion image from the phosphor screen and a spectrum from the multichannel plate are acquired simultaneously. With the current detector limitations either a single *m/z* range image can be acquired by selection of the ion of interest with a set of high-resolution blankers or a total ion image can be acquired by switching the blankers off. Fig. 4 shows an example of this approach. To obtain the images in Fig. 4, three MALDI samples were prepared of 4 peptides (Leucine Enkaphalin, Bradykinin, Luteinizing Hormone Releasing Hormone and Substance P), a synthetic polymer mixture (PEG1500 and PEG3000), and the protein Cytochrome C. These samples were mixed with a solution of the matrix 2,5-DHB and subsequently spotted on a stainless steel surface. While drying, each spot was covered with a 3 mm diameter TEM grid. The surface was examined with MALDI stigmatic imaging while continuously moving the target with 50 μm/s underneath the 150 μm laser spot. A total of 1750 images and 1750 spectra were simultaneously acquired with a repetition rate of 9 Hz resulting in a total acquisition time of 194 s. With image concatenation software that has been developed specifically for this purpose in the Amsterdam virtual laboratory for e-science, all images were overlaid to form the image in the top frame of Fig. 4. From the enlarged image it is clear that the 25 μm hexagonal holes in the grid can easily be observed. The resolving power is estimated to be approximately 2 μm. This result demonstrates both the increase in resolution as in acquisition speed compared to microprobe MALDI with the same field-of-view. In a similar manner, but with repeated line scans the total ion image of a rat brain was obtained. This result, shown in Fig. 5 shows the power of MALDI stigmatic imaging on a SIMS instrument, a result of two MS communities coming together.

4. Concluding remarks: Sociological/cultural aspects

The last aspect that merits discussion is the cultural difference between two science communities, the SIMS community and the biological mass spectrometry community. The SIMS community has long been dominated by physicists and physical chemists with a strong focus on fundamental research of desorption and ionization processes. Applications were mainly found in

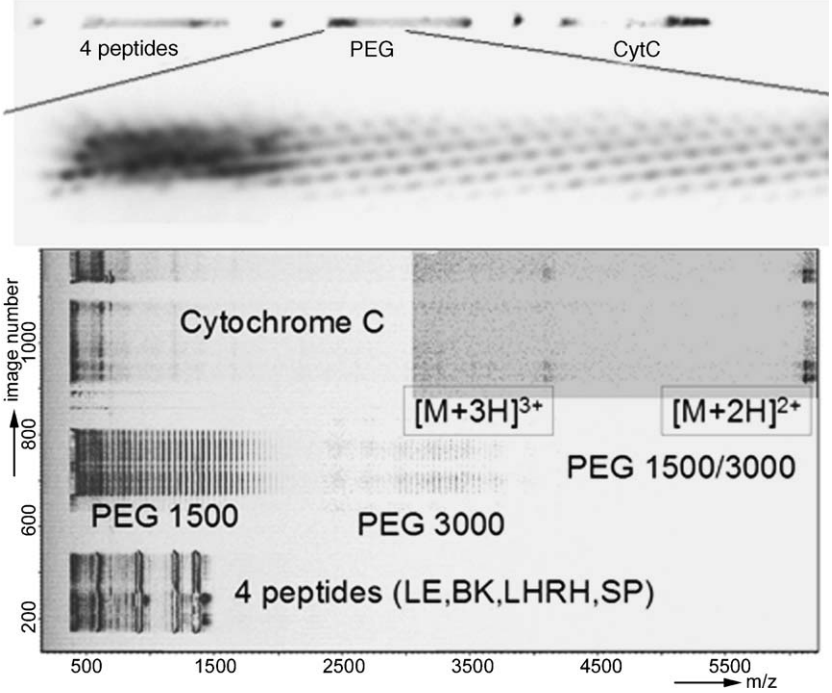

Fig. 4. Stigmatic imaging results from a single line-scan of 3 TEM grids with a diameter of 3 mm. The total image area is 9.7 mm × 150 μm. The grids were placed on three preparations of 2,5-DHB mixed with a 4 peptide mixture, a polymer mixture and a single protein (CytC), respectively. The top image show 1750 concatenated total ion images and the middle image shows a magnification of the dataset. The third panel displays all 1750 spectra that complement these images.

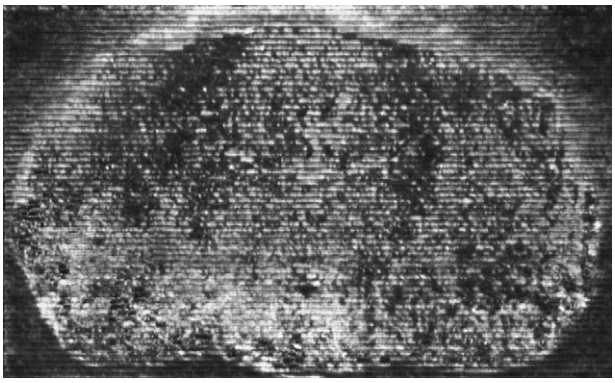

Fig. 5. Stigmatic MALDI image of a rat brain section. The image consists of ~15000 individual images and occupies 70 Gbytes of disk space. Two millimeters size features can still be resolved.

semiconductor research and the majority of SIMS instruments are found at semiconductor manufacturers where they are used for quality control and process monitoring. A strong focus on instrumentation development has greatly advanced the instrumentation in the areas of mass and spatial resolution, sensitivity, speed and field of view. Meanwhile, there has been to be little interaction with the biological MS community that since the conception of ESI and MALDI has focused more and more on high throughput peptide and protein identification and quantitation. Although the fundamental understanding of the different ionization processes is still growing, it is not a major research topic. Application development with techniques that may not be fully understood but that do work and solve real biological problem takes prevalence. In the biological MS community more and more (bio-)chemists become mass spectrometry users that obtain spectra with the push of a button without a thorough understanding of the fundaments of MS. As a result, the MS education shifts its focus towards MS application in the biological sciences. In the corrseponding curriculum SIMS plays no role whatsoever. The perspective from the ever-growing MS industry is apparently different. Vendors of SIMS instrumentation do not strive for an active presence at major MS meetings illustrating that they too acknowledge that that is not their major market. Conversely, mainstream MS vendors do not attend SIMS meetings for much of the same reasoning. Slowly, these communities are searching for common ground. The majority of the 50 or more surface analysis and MS imaging papers presented at the 2005 ASMS meeting dealt with biological surfaces. It is clear that there the future of MS imaging lies, whether with lasers or ion beams.

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