MALDI Produced Ions Inspected with a Linear Ion Trap-Orbitrap Hybrid Mass Analyzer

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A MALDI source is interfaced to a modified LTQ Orbitrap XL instrument. This work gives insight into the MALDI source design and shows results obtained with the MALDI source coupled to an accurate mass, high-resolution hybrid mass spectrometer. MALDI-produced ions and fragment ions thereof produced in the mass spectrometer may be analyzed and detected by the Orbitrap analyzer at a maximum mass resolution of 100,000 (FWHM) at m/z 400 with high mass accuracy. An accuracy of ≤ 2 ppm is achieved by internal mass calibration using lock mass functionality; using external mass calibration, an accuracy of ≤ 3 ppm is routinely obtained. External mass calibration of the hybrid mass spectrometer is performed using a standard calibration mixture of different peptides and matrix components. The instrumental capabilities are demonstrated for analytical methodologies such as Protein ID using Peptide Mass Fingerprint (PMF) and MS/MS analyses of small molecule samples. Stability of mass accuracy and signal-to-noise ratio for low samples loads (on plates) are demonstrated as well as the experimental dynamic range using α -cyano-4-hydroxy cinnamic acid (CHCA) matrix. (J Am Soc Mass Spectrom 2009, 20, 1451–1463) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Since its invention in the 1980s, a matrix-assisted laser desorption/ionization (MALDI) source [1–3] is typically coupled to time of flight (TOF) mass analyzers. Short temporal spread ($\sim$ ns) of ultraviolet (UV) lasers and the small spatial distribution of desorbed ions match the requirements for time of flight mass analysis. Significant advances in fast electronics (data acquisition, detectors, high voltage (HV) pulsers, data processing), ion optics design, and the use of delayed extraction method [4–7] led to the development of research grade and commercial TOF mass analyzers, exhibiting both high mass resolution ($RP \sim 10,000\text{--}20,000$ at FWHM), mass accuracy ($\sim$ down to a few ppm), and also high sensitivity due to low transmission losses [8].

Axial TOF Instrumentation

Still mass analysis required skilled choice of laser energy (normally slightly above threshold for MALDI ion production), use of internal or external standards for calibration placed into the sample or in close proximity to the sample, thermal stabilization, and elaborated sample preparation. Increased laser energy is experimentally proven to help generate more ions and improves statistics

for low abundant peaks, but also normally is accompanied by degraded mass resolution, mass accuracy, and intense in-source fragmentation [9]. Moreover, even though axial TOF systems are capable of recording mass spectra in a wide mass range, it is impossible to achieve uniform mass resolution across the whole mass range because the optimum delay time is mass-dependent. One has to choose between peak mass resolution in a narrow mass range and lower mass resolution attainable over the broader mass range [10, 11] or stitch composed mass spectrum from various segments at the expense of duty-cycle. Efforts to resolve mass-dependency limitation of the delayed extraction method were undertaken by different groups [12–14] but in addition to more complicated electronics, it makes calibration highly nonlinear, requiring the use of standards.

Collisional Cooling Interfaces

Another approach taken is to avoid the direct coupling of MALDI sources to mass analyzers. Maintaining the sample plate at an elevated pressure (ranging from tens of mTorr to atmosphere) [15–19] allows the MALDI event to occur under conditions in which ions' internal degrees of freedom are thermalized before onset of in-source fragmentation [20, 21]. Consequently, one may increase energy delivered to the sample and, as such, number of ions generated per shot by factor of 10 per a 30% increase in laser energy without undesirable

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fragmentation [22]. Following this path, there is no need for high repetition rate lasers (some hundred Hz to ~kHz), if laser operation is at several tens Hz but higher power produces comparable number of ions with much less number of shots during a similar time interval. This is especially true in the view of the superior characteristics of nitrogen lasers, which can only be matched by solid-state lasers when special efforts are made to modify the output beam profile [23]. Also, because sample morphology is not a factor anymore (as it is in an axial geometry) affecting mass resolution, other sample preparation techniques, such as thick-layer, sandwich-type, or relatively thick tissue samples become made feasible in proteomics and other areas.

Another important fact is that having a collision cell with supplementary ion optics allows the use of a broad variety of mass analyzers, including orthogonal acceleration (oa)-TOFs, single and triple quadrupoles, 3D and linear ion traps, FT-ICR, and Orbitrap systems. Even though it may seem counterintuitive to use a collision cell between MALDI source and mass analyzer (minding intrinsic advantages of match in temporal and spatial spreads of MALDI ions to axial TOF), the above mentioned benefits provide solid arguments in favor of such an approach. An orthogonal introduction of a MALDI sample into an ion source extraction region (without collisional cooling) was first introduced for research purposes of studying ion velocities distributions in the MALDI plume [24]. A geometry of a collision cell interface between MALDI source and the TOF mass analyzer, arranged in an orthogonal acceleration configuration (oa-TOF), was implemented and proven efficient in (mass accuracy 30 ppm with external calibration, $m/z < 6000$) repeating the earlier success of a similar arrangement with an ESI source [25].

Hybrid Instrumentation

Having significantly reduced the phase space of the ion cloud and the intact, original composition of the desorbed ions by collisional cooling in the source region gives access to a variety of ways the ions can be handled. Not only direct mass analysis of precursor ions but also their structural information can be derived using combination of collisional cooling and isolation/activation steps. This enables using the principles of modularity in building hybrid systems, justified by the fact that there is no single mass analyzer combining all best characteristics of existing types of mass analyzers. Among the variety of tested hybrid configurations, carrying MALDI ion sources at elevated pressure and MS/MS capabilities, the following stand out: MALDI-3D ion trap-reflectron TOF [26], MALDI-Qq-oa-TOF [27], MALDI-QqQ [28], MALDI-Qq-FTICR [29]. The MALDI-3D ion trap-reflectron TOF system demonstrates high mass resolution and good MS/MS quality but requires a careful choice of the pressure in the cell as compromise between the two devices, the ion trap and the TOF

analyzer. MALDI-QqQ systems have proven good for rapid quantitative measurements on drug molecules and other low molecular weight compounds but tend to be less suitable for proteomics. MALDI-Qq-FTICR systems deliver very high mass resolution and high mass accuracy but have a limited detector mass range, and a poor transmission efficiency to the ICR cell, especially for a wide mass range. MALDI-Qq-oa-TOF hybrid systems meet many requirements of an efficient hybrid systems, such as high efficiency of ion collection in the source region, adequate resolution of precursor ion selection, wide mass range, and high mass resolution and mass accuracy of several ppm. In hybrid systems of this type, the major issue is a poor transmission (about 2%–5%), resulting in losses of sensitivity. Compensation for these losses requires an increase of the total acquisition time and, consequently, results in a lower throughput. Also, the typical mass accuracy is still below standards set by FT ICR or Orbitrap analyzers. In contrast to the above discussed hybrid systems, the ion trap-Orbitrap hybrid analyzer type is characterized by a high transmission (30%–50%) [30]. This is considered an order of magnitude higher than the transmission in an oa-TOF. The high transmission in an ion trap-Orbitrap hybrid analyzer opens a completely new opportunity from an analytical point of view. It is made even more attractive for singly charged MALDI ions because of very high resolving power and mass accuracy of the Orbitrap analyzer in the mass range above m/z 2000.

The work presented here gives insight into the characterization of the instrumental performance of a linear ion trap-Orbitrap hybrid mass spectrometer equipped with a MALDI source making use of a collisional cooling interface. The instrumentation routinely performs accurate mass measurements of MALDI-produced ions in MS and MS/MS mode. Performance criteria, such as mass accuracy, mass resolution, dynamic range, and sensitivity are investigated also by using protein digest samples for identification by Accurate Peptide Mass Fingerprint (Accurate PMF) as well as using samples of small molecules.

Experimental

Mass Spectrometer

A standard LTQ Orbitrap XL instrument has been used for all the experimental work described in this paper. The instrument has an overall mass range of m/z 50–4000, while, e.g., m/z 150–2000 or m/z 200–4000 can be scanned simultaneously in the Orbitrap analyzer [30–32]. The heated capillary used in ESI mode of operation is replaced by the quadrupole q00 for MALDI operation. Ions produced by MALDI are collected in q00 and sent as packages to the linear trap (LT) or further down to the Orbitrap detector. A schematic is shown in Figure 1a. CID of analyte molecules can be performed in the LT, and fragment ions can be detected in the LT or sent

Figure 1. (a) Schematics of the linear trap Orbitrap hybrid analyzer equipped with a MALDI source (MALDI LTQ Orbitrap XL). (b) MALDI source design and schematics of q00. Please refer to the text for further details.

further down to the Orbitrap detector for detection of accurate fragment ion masses. Additionally, the mass spectrometer is equipped with a collision cell to perform higher energy collisional dissociation (HCD); fragment ions are detected in the Orbitrap detector [33]. The collision cell is located behind the curved linear trap (CLT, C-trap). Ions can undergo collisions of low-energy type, which result in triple quadrupole-like fragmentations [34]; for this purpose a precursor ion (m/z ratio) of interest is isolated in the linear ion trap and transferred via the CLT to the collision cell. Fragment ions down to approximately 5%–10% of the precursor ion mass can be observed. This allows detection of immonium and immonium-related ions [35].

MALDI Source

A schematics of the MALDI source is presented in Figure 1b. The MALDI source region includes: a movable sample plate (#1, see Figure 1b), an RF-only quadrupole with auxiliary DC resistive rods (DC AUX) mounted in the gaps between the RF rods (#3). Auxiliary DC rods are made of ceramic (2.0 mm in diameter, 130 mm long coated with resistive material, 120 kOhm per rod). All four rods are bundled together in parallel on both ends. The skimmer (#4) with a 2.2 mm diameter orifice separates the source region held at 75 mTorr from the next pressure region. On the front side, a 4 mm aperture (#2) is placed equidistant from both the RF-

rods assembly and the sample plate, about 1 mm away. Its purpose is to serve as an ion optics element providing a physical barrier between the high RF voltage of the quadrupole rods and the sample plate, and it also carries a DC voltage to shape the ion cloud and focus ions into the q00 region. The upper limit of the aperture diameter (4 mm) was chosen not to obstruct the laser beam without further complication of the aperture design, such as transparent windows or meshes. The ion optics downstream of the MALDI source is identical to that in the electrospray instrument starting from ~ 1 mTorr region optics (#4) and down to the Orbitrap mass analyzer. Sample plates are of standard micro-titer plate (MTP) format, e.g., 384 spots per plate, made of stainless steel; for more details on plate types, see below. The plate is locked on the top of the base plate (holder) equipped with mounting features to transfer between atmosphere, an intermediate pressure chamber, and the ion source region. Movement of the sample plate inside the ion source is provided by a high-precision x-y lead-screw mechanism driven by stepper motors with position accuracy of about 1 μm . Having an interlocked intermediate pressure chamber allows the system to always maintain the source region at low-pressure. The two-stage chamber equipment eliminates the need for venting the system or placing an elaborate valve mechanism in the ion beam path when loading the sample plate.

Optical Layout

Ions are generated using a commercial N_2 ($\lambda = 337$ nm) laser (model MNL-100; LTB Lasertechnik Berlin GmbH) with a repetition rate up to 60 Hz, a maximum energy per pulse of 75 μJ , a laser beam size of 4×2.5 mm, and a 3 ns pulse duration. A direct beam configuration is used in which only two adjustable flat mirrors and a single plano-convex lens ($f = 125$ mm) are required to align a focused laser beam onto the sample plate. The incidence angle of laser beam (beam relative to the sample plate normal) is 32° . The laser beam passes through the narrow ellipse-shaped opening in the RF rod and through the aperture separating the sample plate from the RF-only multipole (q00), see Figure 1b. Similar openings are present in the remaining RF rods for the purposes of light transfer for sample illumination and to help balance the RF quadrupole field. A second flat mirror in the optical path is dichroic to pass visible light from the illuminated sample to the CCD camera. The on-screen captured image magnification is about $50\times$.

The LTB laser control firmware allows attenuation of the output energy in a step-wise manner with 250 steps in total. To provide finer energy adjustment, two additional neutral density (ND) filters (o.d. = 0.3 or $2\times$ attenuation per filter) are installed in the optical path, see Figure 1b. The ND filters can be removed one-by-one when laser energy gradually goes down, approaching the specified life-time limit, about 60 million shots.

Removing the ND filters will increase the lifetime of the laser cartridge well beyond this specified limit.

Operation of the Instrument

Mass spectrometric instrumentation in which ion source and detection is decoupled, as in oa-TOF or an ion trap based instrumentation, can work at elevated laser energies, thus generating much more ions per laser shot without inducing excessive in-source fragmentation or impeding mass resolution of mass analyzer. In case of Orbitrap detection, in particular, up to 10^5 – 10^6 charges can be analyzed per single scan event. For typical sample amounts on the plate, such high number of charges can be achieved with only a few number of laser shots; most of data shown in this article are acquired with 5 to 50 laser shots per single full scan or MS/MS scan.

The choice of the ion source pressure for collisional cooling is a compromise between multiple factors. Higher in-source pressure helps to preserve softness of the desorption/ionization process while, above a certain limit, an increasing risk of excessive clustering and ion losses in the interfacial region is likely to occur. Also, transfer times are increased proportionally with pressure. Setting pressure below the optimum poses the risk of excessive fragmentation, especially at an elevated laser energy that must be avoided when analyzing compounds with labile functional groups, such as phosphopeptides. In this work, ion source pressure is maintained at 75 mTorr; this is found to be a viable compromise between requirements of low in-source fragmentation and overall speed of ion transfer.

A pull-push sequence of the applied axial DC field is implemented to improve speed and efficiency. A simple pull-type axial field at first seems a natural choice, but when ions are simply pulled with an axial DC field to the back end of the quadrupole (negative voltage for positive ions), a more negative voltage offset on the back end of the DC AUX rod gives a stronger force resulting in faster ion transfer. However, this happens to be not the optimal choice when operating at high mass range, up to m/z 4000: We noticed that for high m/z ions, the effective restoring force imposed by the main RF quadrupole field on the radial ion cloud expansion gets progressively smaller, and stored ions may escape between the RF rods to the negatively offset DC AUX rods. This becomes even more pronounced when operating with heavy ion loads exceeding several hundred thousand ions, which is typical for Orbitrap operation. A simple increase in RF voltage resulted in an adverse effect on the transmission of low m/z ions (Kovtoun, V. unpublished results). Instead, a two-step scenario has been implemented: first, positive ions are focused into q00 with a pull-type operation; both ends are at negative voltage, with a higher in magnitude at the back end. Subsequently, positive ions are pushed toward the back of q00 by pulsing on a pushing field using a high positive voltage on the front end of the DC AUX rods

and lower positive voltage at the back end of the DC AUX rods. These voltages (positive for positive ions and visa versa) make ions' escape between RF rods energetically unfavorable. A time diagram for this mode of operation is displayed in Figure 2. Another scenario involves trapping of ions in the q00 region before expelling the ions to the linear trap using the skimmer (#4, Figure 1b) as an ion gating electrode. This is different from an earlier contribution [36] in which final mass analysis was performed in a TOF, because ions are collected before the analysis inside the linear ion trap.

It is rather convenient not to synchronize the laser firing to when all ions have left q00, as this would require a delay in firing the laser. The simpler way is to collect and trap the ions for several consecutive laser shots during a single pull-action (low drag force) and then release them from q00 in a single energetic push event. For the described instrumentation, the ion transmission from q00 to the linear trap is estimated to be of 50%. In Figure 2, the difference for single and multiple shots of operation is shown for mass range $< m/z$ 2000; the drag force is lowered because a longer residence time in the q00 region for multiple shots increases the chances to loose ions at the back end and ions are allowed more time to travel through the q00 device. For high mass mode of operation (m/z 200–4000), the time to move ions to the linear trap after the last laser shot is doubled.

Crystal Position System

The implemented crystal position system (CPS) is basically a feedback controlled method between the camera

Figure 2. Time diagram: pull-push mode of operation in the q00. Note that time and voltage drawings are not in scale, refer to the time and voltage values given in the figure.

image (contrast and brightness) and the x-y location on the MALDI sample plate. The CPS methodology enables the source to precisely locate the sample positions on the MALDI plate, i.e., MTP positions, and to automatically locate the center of the individual sample spots, no matter whether this is accurately located in the center of the individual MTP location or not. In consequence, CPS is capable of finding the spots in a liquid chromatography (LC)-MALDI run automatically, because it centers to the center of the sample deposit and not to the center of the MTP location. This is of benefit for LC MALDI applications; LC MALDI spots can be tiny in area and can easily cover less than 1 mm² only (deriving from only 150–300 nL of solvent (matrix and analyte)). It is not atypical for spotters to not accurately position the LC spot in the center of individual MTP location, but to position the spot, e.g., 0.5 mm, off axis. The CPS functionality “sees” the spots, locates the center of the sample, and starts to raster the sample plate according to user-defined settings from the center of the sample deposit. First results can be viewed by the contributions from Vinh et al. [37] and from Blethrow et al. [38].

Automatic Gain Control (AGC)

As it is of importance in ESI mode to assure a known number of charges in the LT (in particular, to not overfill the ion trap), it is of the same importance in MALDI mode. Also in MALDI mode the number of charges (allowed to move into the LT) is determined and monitored before every single analytical scan. This is done in the so-called AGC scan or pre-scan event. This pre-scan is done before an analytical scan, for which the pre-scan serves as a prediction or forecast into the future. In ESI mode this pre-scan is ensured by a snapshot of the continuously flowing current; it is assumed that the electrospray flux (ion current per time) is approximately constant for pre-scan, which injects ions into the linear trap for 1 ms, and analytical scan, which injects ions into the linear trap according to the AGC measurement and user defined number of charges settings (target value). In other words, if the user asks for a target value of 30,000 charges in the ion trap (analytical) scan and the pre-scan (AGC) experiment has determined 10,000 charges for the 1 ms AGC pre-scan injection time, the analytical scan needs to use 3 ms injection time to meet the user's request in number of charges in the linear ion trap.

Such an approach—to forecast the number of ions for the analytical scan by the pre-scan—is also possible in MALDI mode, though shot-to-shot variability of the MALDI process makes the approach more challenging. To mitigate this variability, the instrument control software takes three laser shots from a given sample x-y location, and the resulting ions are sent to the LT to measure the number of charges in these three laser shots; for this purpose an ion package consisting of the three laser shots is collected in q00 and then sent to the

linear ion trap. The average total ion current (TIC) per laser shot is the basis for the prediction of ion flux expected in the analytical scan. Again, if for example the average TIC per laser shot determines 10,000 charges and one asks for 100,000 charges in the Orbitrap experiment, 10 laser shots are provided for the analytical scan right after. Per definition, the analytical scan has to come from the same x-y location as the pre-scan. To improve the confidence in the predicted number of charges per laser shot, more than one laser shot is used in the pre-scan mode to take into account shot-to-shot laser energy variation. An AGC pre-scan is followed by an analytical scan onto this x-y location. Moreover, one may have several pre-scans and analytical scans from a particular micro-spot, dependant on the signal intensity (e.g., as long as the signal meets certain threshold criteria). Under no circumstances a new micro-spot is selected and used with old/previous AGC data. This procedure excludes any sample heterogeneity effects. To “clean up” a micro-spot of a sample expected to be contaminated with salts and buffers, there is an option to fire the laser several times onto each new x-y location before the first AGC pre-scan; this allows stabilizing the signal before the AGC evaluation. The typical variation (2σ) in output laser energy is about 4% in continuous operation and may exceed this value in burst mode when interruption in firing occurs while ions are scanned out of the LT.

Another distinction from the ESI source is the violation of a simple linear dependence between number of laser shots and accumulated ion population (in ESI source this would be identical to a nonlinear dependency of ion population in the LT versus injection time. For example, for the conditions used for instrument calibration (see below), ion population may increase faster with additional laser shots than predicted by the pre-scan evaluation, and later the trend may reverse when the sample is continuously consuming at this given x-y location [39, 40]. This effect may be sample-, matrix-, preparation-, or laser energy-dependent. To appropriately take care of the MALDI phenomenon that the ion current per laser shot can potentially first increase and then decrease [40, 41], the instrument control software does not allow for more than 80 laser shots onto a given location under AGC in Orbitrap detection. Spoken with ESI and LT terminology, the “maximum ion injection time” is reached if 80 laser shots are requested. A new AGC scan would then be needed; this can be obtained from the identical x-y location or from a new location next to the previous one; the functionality can be set by the operator. In addition to the AGC functionality, it is possible to set the number of sweep shots (to be discarded) made onto a given x-y location before the first AGC evaluation onto this position.

In case the number of ions generated per single laser shot exceeds the requested number of charges (target value), the ion beam is attenuated before enter the LT using the ion optics. This approach is preferable over

lowering of the output laser energy, especially when sample consumption is not an issue, because below a certain level of energy the number of ions generated per laser shot shows significant fluctuation, even with high load of sample on the plate. Moreover, it is known from the literature [41] that dependence of MALDI ion output on laser flux is highly nonlinear, especially at low fluxes [42].

The requirement for ion beam attenuation by ion optics obviously is to provide the lowest possible m/z discrimination across the broad mass range. The DC value on the lens separating the inner volume of the LT from the q1 region (octopole in front of the LT, Figure 1a) is used for this attenuation; the so-called “front lens” is automatically varied for attenuation. Results of such attenuation are shown in Figure 3 for ions covering the mass range m/z 524–3659. Typically, not more than a factor of 10 $\times$ attenuation is available (voltage range 2.5 V), and otherwise m/z discrimination favoring higher m/z ratios occurs. This attenuation method typically shows less than 25% discrimination across the mass range.

Taking all the above aspects into account, the AGC technique allows populating the LT in a wide range of user-defined target values if both tools, number of laser shots, and ion optics beam attenuation are employed. Asking for a target value of 30,000 charges, 95% of the data are acquired with a final ion population in the range of 10,000–30,000 charges, even though the number of ions (charges) per laser shot varied by almost three orders of this value. The described experimental evidence is shown in the graph provided with Supplemental Figure I, which can be found in the electronic version of this article.

Instrument Calibration

All devices of the instrument are calibrated and tuned with MSCal4 from Sigma-Aldrich, St. Louis, MO, USA, ProteoMass MALDI Calibration Kit for LTQ XL, and LTQ Hybrids. The kit contains various peptides and α -cyano-4-hydroxy cinnamic acid (CHCA), and is ready-to-use. All peptides used for mass calibration are shown in Table 1 (provided in the Supplemental Infor-

Figure 3. Dependence of ion beam attenuation by variation of the DC offset on the front lens.

mation) along with their sequences and accurate ($M + H$)⁺ masses. More details on the calibration kit can be found in the data sheet from MSCal4 at <http://www.sigmaaldrich.com/sigma/datasheet/MSCAL4dat.pdf>. To mass calibrate Orbitrap's high mass range m/z 200–4000 in positive ion mode, the CHCA matrix dimer ($2M + H$)⁺ at m/z 379.09246 plus peptides at m/z 524.264964 (peptide MRFA), m/z 1060.568674 (bradykinin), m/z 1936.985048 (ACTH 1–16), m/z 2845.761447 (mellitin), and m/z 3657.928859 (ACTH 7–38) are used as reference masses.

As in ESI mode, in MALDI operation also, various numbers of ion charges (target values) are requested automatically, and mass calibration is achieved for the given target requests. Upon mass calibration the mass deviation (in ppm) is measured for the various Orbitrap cell loads. The higher the cell load, the more ions exist in the Orbitrap per given event and the more they influence each other by Coulomb repulsion, the more the mass measurement deviates from the correct mass. The slope of the curve is used as the basis for later analytical measurements and to compensate for the mass deviation upon high (10^5 – 10^6 charges) target requests.

If not otherwise mentioned, all spectra displayed in this contribution make use of external mass calibration using CHCA matrix. Typically the mass calibration is about 0.5 to 1 d old or older. There is no need to have calibration spots on the sample plate of investigation; mass calibration of the Orbitrap analyzer can be performed earlier and on another plate, and is decoupled from the analysis itself.

Sample Preparation

Sample preparations used in this study follows two basically different approaches: (1) fast evaporation or thin-layer strategy [43, 44] and (2) dried droplet technique [45–47]. As it is well described in the literature [48], less water-soluble matrices [49–51] are likely to be used for thin-layer preparation, while better water-soluble matrices are likely to be used in the dried droplet preparation techniques [45, 52]. Both approaches were successfully applied in this study using the described and published protocols.

To allow for a most effective MALDI process (effective in terms of laser fluence needed in relation to the TIC measured), a laser irradiance of the matrix solid close to the maximum absorption of the matrix solid is beneficial. Most common UV-MALDI matrices for desorption at laser wavelengths 337 nm (N_2 laser) or 355 nm (frequency tripled Nd:YAG laser) show an absorption band with a maximum closer to 337 nm, even if the absorption shifts toward higher wavelengths for the solid-state (relative to the solution absorption) is considered [53–55]. It is further understood that the nitrogen laser beam provides the best choice for UV-MALDI applications [23] with regard to beam profile over and sample ablation from the irradiated area.

In this work, CHCA matrix and 2,5-dihydroxybenzoic acid (2,5-DHB) are used as matrices and were purchased from Sigma Aldrich or from LaserBioLabs (Sophia-Antipolis, France) and prepared according to protocols referenced above. With these two matrix compounds, a known “hot” (CHCA) and a known “cold” (DHB) matrix are used for the analyses [56]. For CHCA matrix, a solvent consisting of acetone [43] or high content of acetonitrile plus ethanol (recipe of MSCal4, see above) is used with a final matrix concentrations of <3 g/L. For 2,5-DHB matrix a basically (mostly) water-containing solution of a concentration of <10 g/L is prepared.

For studies and analysis of samples deposited on Qiagen Mass Spec Turbo Chips (for their functionality see www1.qiagen.com) two different samples are prepared: (1) Angiotensin sample (sequence DRVYIHPF, ($M + H$)⁺ 1046.541791) provided as single peptide sample (part of the MSCal4 Kit) is quickly prepared in a dilution series from 1 pmol/ μ L down to 5 and 1 amol/ μ L. One μ L deposits (each) of the different dilution steps are loaded onto the Turbo Chips and allowed to dry; (2) an enzymatic digest of bovine serum albumin (BSA) is available as a stock solution (1 pmol/ μ L, 20 μ L total volume) in water containing 0.1% trifluoroacetic acid. A dilution series down to 100 amol/ μ L and 20 amol/ μ L is prepared and deposited quickly in 1 μ L volumes on the Mass Spec Turbo Chips. All preparations are completed with a short (2 s long) washing step applying the “finalizing solution,” (for further details refer to the Mass · Spec · Turbo Chip Handbook) [57].

For the analysis of small molecule samples, a mixture of atropine, sulfadimethoxine, buspirone, reserpine, and its saturated by-product is analyzed with CHCA matrix. For sum formulae and accurate ($M + H$)⁺ masses of the compounds, refer to Table 2, provided in the Supplemental Information. A dilution series is explored with the final concentration of each compound of 500 fg/ μ L prepared on a stainless steel MALDI plate.

Sample Plate Holders

Various sample plates can be used, which can be basically differentiated in two types: MTP formats (96, 384, and 1536 on stainless steel basis), and plates for imaging applications in which a continuous scanning across the deposited sample is of interest. The latter plates can carry (not necessarily conductive) glass slides or metal slides; these can be readily used for tissue imaging applications. In any case, the sample plate (footprint is according to MTP format) is fixed on a base plate holder; this single adapter plate carries different kinds of plates provided.

Besides the above mentioned sample plate types, plate holders to carry Qiagen Mass Spec Focus Chips (Qiagen, Hilden Germany) and Qiagen Mass Spec Turbo Chips are available. For details on the Qiagen Mass Spec Focus or Mass Spec Turbo Chip functionality, refer to the corresponding homepage.

Results and Discussion

Using the standard calibration mixture (see Table 1 provided in the Supplemental Information), mass accuracy is found to be well below 3 ppm (Figure 4). This is demonstrated for full scans (Figure 4a and b) and for MS/MS scans (Figure 4c). Monoisotopic masses of the peptide isotope clusters are labeled and expressed with respect to deviation from the theoretical masses in Figure 4a. The single full scan spectrum from m/z 200–4000 is a result of three laser shots asking for 5×10^5 charges. The isotope pattern of mellitin (middle part of Figure 4b) is nicely displayed in both single scans (bottom) and in an average of several scans (top). The single FTMS full scans typically request 5×10^5 or 10^6 charges, respectively, and needed only a very few laser shots to meet the user's requested target value. It should be noted that the spectrum acquired at 100,000 nominal resolving power (at m/z 400) demonstrates a resolving power for mellitin of about 50,000. This is significantly higher compared, not only with all MALDI-TOF instruments but also with a 15 Tesla FT ICR system, with the same duration of acquisition time (1.5 s detection time). In case, e.g., a resolving power of 30,000 (at m/z 400) is selected, mellitin (m/z 2845) is seen with a resolution of 11,000 (all resolution power values reflect full width at half maximum, FWHM).

Figure 4c shows a HCD spectrum of the ion displayed in Figure 4b (mellitin) and demonstrates almost complete b- and y-series, accompanied by internal fragments. For clarity reasons, only some of the internal fragments are labeled, see bottom left part of Figure 4c. The HCD technique is chosen here to demonstrate that also singly charged peptide ions of considerable mass can be fragmented, and sequence information is provided. As observed earlier for HCD coupled to more highly charged ions derived from an ESI source, the peptide is fragmented along the entire backbone [34, 35].

Dynamic Range Using CHCA Matrix

Experiments were performed to test the dynamic range of the instrument. According to earlier investigations with the ESI system [58], it is known that the linear ion trap-Orbitrap instrumentation has a dynamic range of 5000/1 in single scan acquisition. Such high dynamic range is typically very difficult to achieve with conventional MALDI-TOF instrumentation, especially in a 1-s acquisition (this compares with 60,000 resolving power at m/z 400 in Orbitrap operation), and cannot straightforwardly be expected with the MALDI technique per se, because the presence of matrix clusters along the entire mass scale degrade the dynamic range [59]. For MALDI TOF measurements, it is well described that every single m/z ratio is occupied, and it is assumed that these peaks result from surviving matrix clusters. The occupation of every m/z ratio can be observed clearly upon averaging; this is considered to contribute to a relevant "noise" band in MALDI TOF instrumentation.

It is assumed that these matrix clusters (at least) survive the flight time from source to detector (some 10 μ s to a few 100 μ s). Additionally, the high resolving power of FTMS instrumentation allows resolving many of such peaks and, thereby, decreases the chemical noise in any particular band significantly [60].

It was investigated by Hillenkamp et al., (personal communications) that the dynamic range also in an oa-TOF instrumentation could not be significantly improved, despite of the thermalization effect in the first quadrupole, neither for UV MALDI nor for Infrared (IR) MALDI.

The spectrum displayed in Figure 5 presents a single scan spectrum from four laser shots requesting 5×10^5 charges for the Orbitrap full scan. The ^{13}C isotope of the phthalate ion at m/z 392.28754 shows up with an intensity 5000 times lower than the base peak intensity at m/z 1060.56,921. This suggests that the dynamic range in MALDI in fact can be significantly higher than earlier experiments with TOF technology suggest. We expect the reasons for this higher dynamic range in the given instrumentation to be: (1) thermalization in the source allows the use of much higher laser energies and hence produces much higher ion currents, (2) very high space charge capacity of the Orbitrap analyzer (limited currently by the C-trap, CLT), (3) possibly collisions with nitrogen bath gas break up matrix clusters in the CLT, and (4) the significantly longer time scale (relative to TOF technology) for the ions to travel from source to detector (ms instead of μ s) could also contribute to decay of matrix clusters.

The obvious noise band, displayed in the inset of Figure 5 with the dotted line, at 0.01% (or $1/10^4$) of the base peak intensity, originates from the thermal noise of the image current pre-amplifier: At the mass resolution of this scan (100,000 at m/z 400) the noise band is equivalent to a signal from five elementary charges [61]. This number, multiplied by the number of all peaks including isotopes (summed up to 100% intensity), 10×10^4 (ratio of base peak intensity to background) is in good agreement to the number of charges the experiment requested (5×10^5).

Attomole Peptide Loads on Plate, FTMS, and FTMS/MS Sensitivity Using Angiotensin

To investigate the instrument's sensitivity, samples with very low analyte amounts were prepared. It is well described in the literature [62–64] that substrate surfaces, which allow for a concentration step upon sample drying (typically it is a hydrophilic spot surrounded by hydrophobic environment), are best suited for these sensitivity experiments.

Our investigations using Mass Spec Turbo Chips show that a peptide load of 1 amol Angiotensin II, placed in a 1 μ l volume onto the matrix deposit of the matrix pre-prepared sample plate, can still be detected with a decent signal to noise ratio, $S/N = 4/1$ to $5/1$.

Figure 4. (a) FTMS full scan, mass range m/z 200–4000 acquiring the MALDI calibration mixture at resolving power 30,000 at m/z 400, three laser shots are used for the single scan displayed, number of charges requested (target value) is 5×10^5 , elapsed scan time is 0.75 s. The mixture contains several peptides (MRFA, bradykinin, ACTH 1–16, mellitin, ACTH 7–36, see Table 1). Mass accuracy is given and described for the monoisotopic masses in the individual isotope patterns. (b) Same mixture, acquisition taken from FTMS full scan using resolving power 100,000 at m/z 400, five laser shots are used for the single scan displayed, number of charges requested (target value) is 1×10^6 , elapsed scan time is 2.37 s. Inset into region of peptide mellitin at m/z 2846. The theoretical isotope pattern (see middle part) is nicely reflected in the measurements; an average of 10 scans and a single scan reflect the isotope pattern and determine the monoisotopic mass of mellitin correctly. The resulting mass resolution at m/z 2846 is approximately 50,000 (FWHM). The spectrum is acquired with external mass calibration performed the same day. (c) Same mixture, MS/MS of the ion at m/z 2846 (mellitin) using HCD. HCD energy setting equals to 220 eV. An average of five scans, total of 100 laser shots is shown. Almost complete b- and y-ion series and various internal fragments are found with a mass accuracy of better than 3 ppm.

Figure 5. Expanded region of a single FTMS full scan information m/z 150–2000 deriving from four laser shots asking for 5×10^5 charges. Analyte molecule: bradykinin, matrix: CHCA, (see Table 1 provided in the Supplemental Information). The inset from 391–392.5 displays two signals at m/z 391, the lower one is approximately 1000 times lower in intensity than the base peak at m/z 1060.56 and can straight forwardly be assigned to phthalate. This ion at m/z 391.28,404 ($C_{24}H_{39}O_4$) is accompanied by its ^{13}C relative at m/z 392.28,754 ($C_{23}^{13}CH_{39}O_4$). The latter shows up with an intensity 5000 times lower than the base peak intensity. A background noise line at 0.01% (1: 10,000) is assigned with the dotted line. Accuracy of mass measurement is shown in the figure.

The spectrum shown in Figure 6a displays a single scan, which is an outcome of seven laser shots only. The investigation is performed (as for all others analyses in this contribution) with AGC “on.” The analytical scan asks for 10^5 charges. The spectrum shown is purposely acquired in full profile mode to demonstrate the band of thermal noise from image current pre-amplifier. Averaging over several scans (10 scans averaged in Figure 6b) from this sample shows clearly that the signal to noise ratio can easily be improved, and also m/z values of the accompanying peaks of the Angiotensin pattern (isotope pattern) are accurately measured, even though being even lower in intensity.

Further experiments are carried out to investigate the possibility to get sequence information of very low peptide loads on the sample plate. For this purpose, a 5 amol load (1 μ l volume) of the same Angiotensin source is prepared onto the Qiagen Mass Spec Turbo Chips. Fragmentation is carried out using HCD technique and “stepped collision energy” functionality [33]. In the latter, the ion population is divided in more than one part (2–5 fills, user-definable), and subsequent fills of ions are injected into the collision cell at various collision energies, e.g., one “sub”-scan is acquired at low collision energy, one at medium energy, and one at high collision energy. The resulting final Orbitrap scan combines all three scans into one and shows a nicely balanced HCD spectrum, showing the precursor ion (lower collision energy), fragments in the middle part of the spectrum, and, finally, fragments in the low mass range such as immonium and immonium-related ions (higher collision energy). Figure 6c shows an average of two (final) scans, each a result of three different HCD energies; it is a result of 100 laser shots, asking for $5e4$ charges in each scan. This allows detecting immonium

ions of His (m/z 110) and Tyr (m/z 136). They can be assigned unambiguously due to the accurate mass measurement.

Attomole Peptide Loads on Sample Plate, Accurate Peptide Mass Fingerprint

To investigate low peptide loads for more realistic, more complex samples, an enzymatic digest of bovine serum albumin (BSA) is used for protein identification, using the accurate peptide masses of the proteolytic fragments. Supplemental Figure IIa demonstrates a single-scan spectrum obtained in three laser shots from 100 amol loading. The peak list of accurate monoisotopic ($M + H$)⁺ masses is sent for protein identification by PMF. Protein coverage reached 47% with all peptides being better than 3 ppm accurate. Supplemental Figure IIb demonstrates an average of five scans obtained in 14 laser shots from 20 amol loading. Protein coverage reached 28% with all peptides being better than 3 ppm accurate. Supplemental Figure II is provided in the Supplemental Information.

Small Molecules

The analysis of small molecules with MALDI is of particular interest with Orbitrap detection because matrix background is accurately detected and nicely resolved due to high-resolution, and allows for an easy differentiation from peaks of interest. The current image detection in the Orbitrap analyzer is free from dead time or any saturation effects; the base line of the mass spectrum is flat also for very low analyte concentra-

Figure 6. (a) Expanded region of the FTMS full scan analysis of a 1 amol Angiotensin II (sequence DRVYIHPF, (M + H)⁺ 1046.541791) load from m/z 850–2000, a 1 μ L volume deposited onto a Qiagen Mass Spec Turbo Chip. Single scan deriving from seven laser shots asking for 1×10^5 charges (target value). The spectrum is acquired in full profile mode to entirely demonstrate the “noise” band. The right hand side inset into the region from m/z 900–1200 is spread in y-axis intensity and shows that the Angiotensin signal measured in the single scan contributes by about 20 to 25 charges. (b) Inset into the peak of interest of the mass spectrum displayed in (a), m/z 1045–1051. Here an average of 10 single scans, deriving from 73 laser shots, is shown and compared with the theoretical isotope pattern of Angiotensin. The A, A + 1, and A + 2 peak of the Angiotensin isotope pattern are measured with accurate mass. (c) FTMS/MS spectrum (average of 2 scans) of m/z 1046 (Angiotensin) using HCD technique. Five amol of sample loaded onto a Qiagen Mass Spec Turbo Chip, sum of 100 laser shots asking for 5e4 charges per scan. First mass for MS/MS experiment was m/z 100. This allows detecting also immonium ions of His and Tyr. The HCD experiments make use of “stepped collision energy” functionality. For details, please refer to the text.

tions. Supplemental Figure III shows full scan and MS/MS spectra of the small molecules mixture shown in detail in Table 2, see Supplemental Information. The load onto the sample is 500 fg (1 μ L) per compound. The full scan information shows matrix signals and the compounds of interest. Latter ones are explained with respect to mass accuracy also in Table 2. Supplemental Figure IIIb shows the MS/MS information of the two signals at m/z 607 and 609 (see inset in Figure IIIa for the corresponding full scan information). Despite of the fact that all (low concentrated) compounds are low in intensity, both compounds, m/z 607 and 609, can be isolated in the LT separately from each other. Here the narrow isolation capabilities of the LT are used. An isolation window of 1.5 u (± 0.75 u) is applied to each precursor ion. After isolation in the LT, the precursor ions undergo fragmentation by CID, and fragments are sent to Orbitrap detection. As it was known from earlier experiments, the two related compounds at m/z 607 and 609 (unsaturated by-product of reserpine at m/z 607 and

reserpine at m/z 609) can be distinguished by their MS/MS spectra; this is illustrated in Supplemental Figure IIIb by MALDI data. Note that the spectra display a single scan each as a result of 70 laser shots asking for 10^5 charges. The MSMS spectrum of buspirone (m/z 386, see Table 2 and Figure IIIa, all Supplemental Information) is shown in Supplemental Figure IIIc. Here HCD is chosen as fragmentation technique. Peak and structure assignment is a result of MassFrontier software [65]. It is interesting to note that all peaks of the triplet peak series at m/z 148, 150, and 152 are of equal height in HCD mode, while the peaks at m/z 148 and 152 are of considerable lower intensity in the CID spectrum (data not shown). This is to say that the HCD technique can possibly also enable an increase of structural information about small molecules, as it has proven for peptides already. It is speculated that the softness of fragmentation in the linear trap versus presumably much harder (however still low-energy collisions) triple quadrupole like fragmentations in the

HCD cell lead to this result. Supplemental Figure III is provided in the Supplemental Information.

Conclusion

A MALDI source is coupled to a conventional linear trap-Orbitrap hybrid mass spectrometer; the instrumentation is described here with respect to its performance. It has been demonstrated that the detection using the Orbitrap takes advantage of much greater ion numbers obtained by each laser shot and each single scan than provided for typical TOF instrumentation. This is facilitated by de-coupling the MALDI process from the analysis by employing a trapping buffer. In particular, it has been demonstrated that the resulting instrument retains all high-performance characteristic for its electrospray ion source version. Robust MALDI ion production is provided by the presence of gas in the interface; this allows for high ion currents and, consequently, for a high dynamic range. Sensitivity for standards is shown in low amol sample loads on plate while maintaining mass accuracies in the low ppm range. Information-rich, complementary MS/MS spectra are provided by CID and HCD fragmentation techniques. HCD technique applied to singly charged MALDI-produced peptides shows fragmentation over the entire peptide backbone as it is known and documented for multiply charged ESI-produced peptides. For peptide precursors $< m/z$ 2000, significant immonium ion information (m/z 100–200) can also be expected. Peak lists of monoisotopic, accurate peptide mass information of protein digests can readily be used for protein identification (of appropriate sample complexity) due to the accurate mass of all peptides. The high-resolution precursor ion isolation capability of the linear ion trap allows isolation and fragmentation of species that differ by a mass of two hydrogen atoms in individual MSMS spectra.

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2009.04.013](https://doi.org/10.1016/j.jasms.2009.04.013).

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