

ATMOSPHERIC PRESSURE MALDI

MS is a leading technology for proteomics, primarily because of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). These technologies can determine the amino acid sequences of proteins; find the location and detailed structures of posttranslational modifications; characterize DNA alleles (in multiplex) using probes of different mass; and observe noncovalent interactions of proteins with DNA, substrates, or other proteins. Atmospheric pressure (AP) MALDI is a new method that operates outside the vacuum like ESI, and indeed it has some features of both techniques. We expect that AP MALDI will be used increasingly in the MS instrumentation being developed for proteomics and other biological studies, particularly in instruments designed for high-throughput analyses.

ESI and MALDI

MALDI and ESI are, in many ways, complementary techniques. MALDI is used for solid samples (stimulating the development of sample microarrays for MS), electroblotting proteins from two-dimensional (2-D) gels onto membranes that are inserted directly into the instrument, and sample affinity/capture surfaces such as those used in surface-enhanced laser desorption/ionization. ESI works with samples in solution and is well known for its success in coupling liquid chromatographs to mass spectrometers. The time-of-flight (TOF) mass spectrometer is the most logical choice for MALDI, which is a pulsed technique. This has, at long last, earned a place for the mass analyzer, which was introduced more than 50 years ago.

The marriage between ESI and quadrupole mass analyzers has been a good one—the

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A new method for proteomics and other biological studies requiring high-throughput analyses.

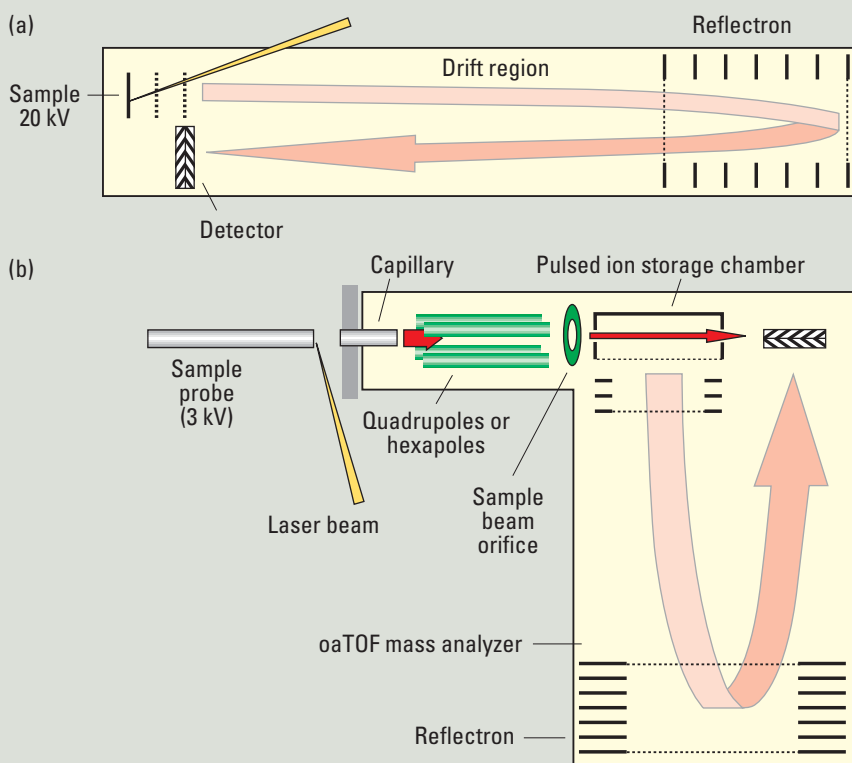


FIGURE 1. Geometries of (a) vacuum MALDI and (b) AP MALDI on a TOF mass spectrometer.

quadrupole is compatible with the continuous ion currents produced by an ESI source, and the multiply charged ions formed by ESI have been a boon to mass analyzers with a relatively modest m/z range. Moreover, quadrupoles can handle the elevated ion pressures from ESI sources. In contrast, TOF mass analyzers need ions accelerated to a precise, constant kinetic energy and typically run into problems from ion collisions at even moderate pressures, despite a series of skimmers and differential pumping regions for reducing pressure. The quadrupole mass analyzer and ion trap do not depend on ion kinetic energies for accurate mass measurements.

The recent development of orthogonal acceleration (oa) has made it possible to couple a TOF mass spectrometer with an ESI source. Guilhaus introduced oa on a mass spectrometer with an electron impact (EI) source, and Dodonov et al. later applied it to ESI (1, 2). The performance of the ESI oaTOF mass spectrometer is improved by the addition of a quadrupole ion guide (3, 4). The ion guide provides an interface between the AP ESI source and the vacuum, and it collimates the ion beam for injection into the mass analyzer. Ion guides are now also used on ion trap and hybrid quadrupole-TOF instruments.

This successful combination of ESI with TOF, ion trap, and tandem (hybrid) mass spectrometers suggests that these might all be compatible with an AP MALDI source. An AP MALDI source would have particular advantages for the high-throughput screening analyses required to build both genomic and proteomics databases, because the sample arrays and positioning

stages would be located outside the vacuum and alongside the associated robotics and process lines. Unlike in the more common vacuum MALDI TOF configuration, mass resolution with AP MALDI would not be plagued by the initial kinetic energy and spatial distributions of the ions. Samples could be presented in volatile solvents, on nonconducting glass slides, or as whole cells or tissues. Moreover, an AP MALDI source could be used interchangeably with any ESI instrument.

AP versus vacuum MALDI

In a MALDI TOF instrument, the ions are formed on a flat surface or probe held at high voltage V which is commonly ~ 20 kV. A short extraction region then accelerates the ions to the same kinetic energy eV and into a long drift region. In a simple linear instrument, the mass m of an ion is then determined from its flight time t in the drift length D by

$$t = \left(\frac{m}{2eV} \right)^{1/2} D \quad (1)$$

This is only approximate because flight times are sensitive to the initial kinetic energies U_0 of the ions that give rise to a distribution of final energies $eV + U_0$, which result in broader peaks and lower mass resolution. In addition, if ions are formed in different locations in the source, then the combined U_0 and ion spatial distributions produce a more complex description of the flight time of an ion, given by

$$t = \frac{(2m)^{1/2}}{eE} [(U_0 + eEs)^{1/2} \mp U_0^{1/2}] + \frac{(2m)^{1/2} D}{2(U_0 + eEs)^{1/2}} \quad (2)$$

in which E is the electric-field strength in the ion source and s is the ion's position in the source.

The reflectron, introduced by Mamyrin (5) and shown in Figure 1a, does compensate for the spread in U_0 but not for the initial spatial distribution of ions in the source. In 1955, Wiley and McLaren introduced a dual-stage extraction time-lag focusing scheme to address the spatial and kinetic energy distributions (6), but the energy correction was mass-dependent. Because it is not possible to fully compensate for both U_0 and position simultaneously, most current schemes begin by eliminating one of these distributions.

In the vacuum MALDI instrument shown in Figure 1a, the formation of ions from a flat and equipotential surface by MALDI eliminates the spatial spread. The initial kinetic energy distribution is then addressed by using a reflectron and/or by delayed extraction (7-9). Delayed extraction is the term most commonly used today for Wiley-McLaren focusing in the absence of an initial spatial distribution, and it has produced extraordinary mass resolutions for MALDI TOF instruments. Still, it is

mass-dependent, focusing only a portion of the mass range at one time, although nonlinear, mass-correlated extraction schemes are being developed to address this problem (10, 11).

Despite the extraordinary results that are obtained from commercial high-performance MALDI TOF instruments, mass accuracy and mass resolution remain sensitive to sample thickness and morphology, variations in the sample surface or position, and laser power. In the conventional MALDI TOF instrument, the mass analyzer “remembers” all of these initial conditions.

AP MALDI. The initial source described by Laiko et al. was interfaced to a PE BioSystems Mariner oaTOF mass spectrometer (12, 13). Their AP MALDI source was a simple, angled probe tip, from which ions were desorbed into a stream of nitrogen gas at the entrance of the inlet nozzle to the mass spectrometer. The source was interchangeable with the ESI source; however, unlike the ESI source, it produced predominantly singly charged ions. The coupling of the AP MALDI source to the oaTOF was possible only because this instrument was designed to accommodate an atmospheric pressure source.

In the oaTOF instrument, it is the U_0 distribution that is eliminated from the focusing problem. More accurately, the velocity distribution in the direction of the TOF axis is reduced or eliminated by collimating the ion beam by a combination of skimmers, orifices, and/or ion guides into an ion storage region (Figure 1b), from which the ions are extracted (accelerated) in an orthogonal direction by a pulsed electric field that focuses the remaining ion spatial distribution (corresponding to the thickness of the collimated beam). At the space focus plane, ions of the same mass catch up with one another, and the spatial distribution is then converted to a kinetic energy distribution that is focused by the reflectron (14).

Note that the spatial distribution in the ion storage chamber and kinetic energy distribution focused by the reflectron are not initial conditions of the ionization process, but result from collimation and extraction of the ions. Unlike traditional TOF instruments, the oaTOF analyzer has no memory of the initial conditions or what ionization method was used to create the ions. For that reason, the oaTOF mass spectrometer has been well suited for atmospheric methods such as ESI, and it should be equally well suited for AP MALDI.

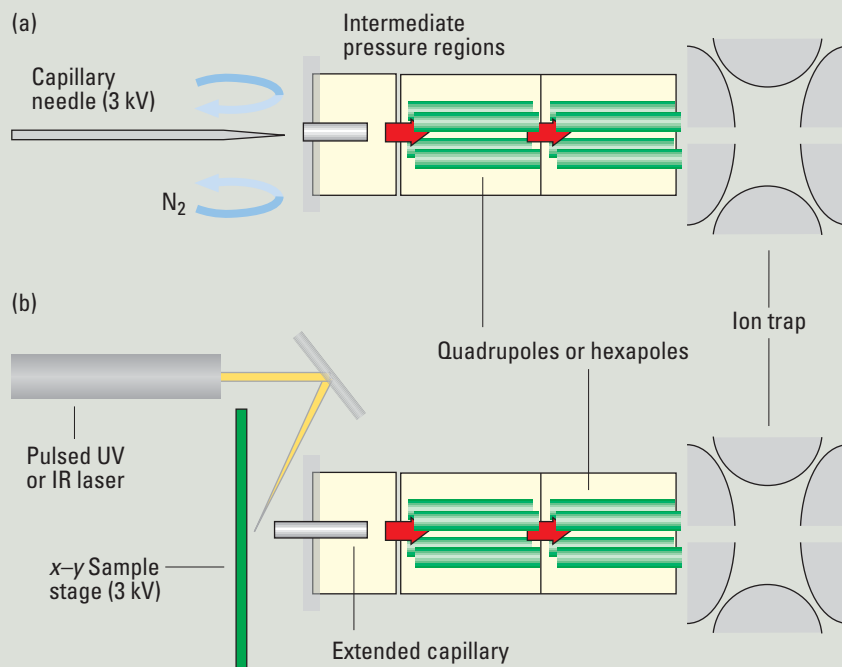


FIGURE 2. (a) ESI and (b) AP MALDI with an ion trap mass spectrometer.

**This combination of ESI with TOF,
ion trap, and tandem instruments
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Ion guides. When skimmers and apertures alone are used to form an ion beam, they do so by excluding all ions with off-axis components of velocity. The quadrupole ion guide focuses these divergent ions and creates a beam that includes many more of the ions from the source. As shown in Figure 1b, the ion guide is located behind the initial aperture (or capillary) that separates the mass analyzer from the atmospheric side, but in a region in which background pressures of a few millitorr provide collisional cooling of the ions. The radio frequency (rf) field then confines and collimates the ions.

Quadrupoles, hexapoles, and octopoles, operated in rf-only mode, have been used as ion guides on oa and hybrid quadrupole-TOF instruments. They are also used in ESI ion trap mass spectrometers, where they reduce ion loss as the ions are transmitted from the mid-pressure region to the mass analyzer.

AP MALDI and the ion trap

Several groups have now combined AP MALDI with commercial ion trap mass spectrometers (15–18). This configuration brings MS^n capabilities to MALDI, and, together with the more easily interpretable singly charged species that this system generates from complex mixtures, it is expected to offer advantages for obtaining structural information from peptides and

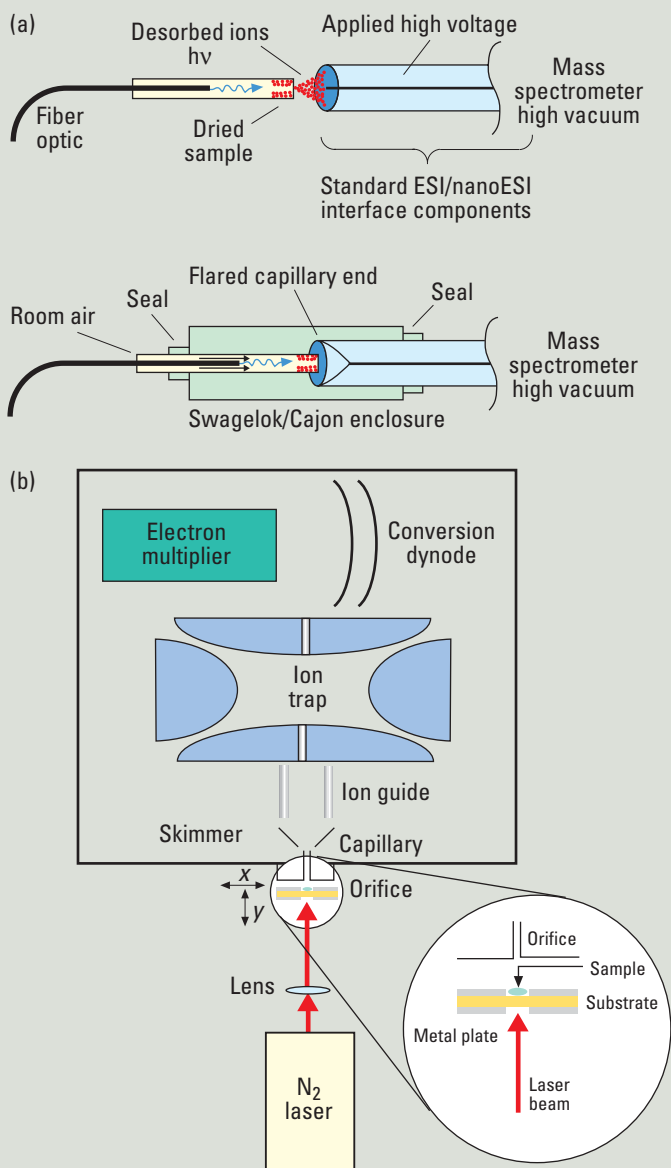


FIGURE 3. Transmission mode AP MALDI ion sources of (a) Danell and Glish and (b) Callahan and co-workers.

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protein digests and identifying and characterizing posttranslational modifications.

Our initial AP MALDI source was a probe configuration similar to that used for the oaTOF, but it was coupled to a ThermoFinnigan LCQ Classic ion trap mass spectrometer with the MALDI probe replacing the ESI needle (12, 15). A frequency-tripled (355 nm) Nd:YAG laser focused onto the surface of the sample probe generated ~ 0.3 -mJ pulses at 10 Hz and was operated asynchronously with the trapping cycle of the ion trap mass spectrometer. A potential of 2.7 kV was applied between the probe tip and the LCQ inlet transport capillary, and dry nitrogen was applied through a stainless steel capillary to the region surrounding the target. The transport capillary was heated

to 180 °C. Ion trapping times were 200–400 ms, so that ions were accumulated over several laser pulses. Because the analytical scan is considerably shorter, the duty cycle is high even though the instrument is run asynchronously. We have reported detection limits of 10–50 fmol of analyte deposited on the target surface for a four-component mixture of peptides with molecular weights ranging between 800 and 1700 Da (15).

Ion trap mass spectrometers accommodate ESI and atmospheric pressure ionization (API) by utilizing a capillary inlet as the interface to the region of intermediate pressure inside the vacuum chamber (Figure 2a). One or more quadrupole, hexapole, or octopole ion guides and differentially pumped regions then provide the transition to the high-vacuum region around the ion trap. Because this arrangement is insensitive to the means by which ions are formed outside the instrument, it is relatively easy to design an AP MALDI source that is completely interchangeable with the ESI source (Figure 2b).

To accommodate the laser beam in the reflection mode as shown in Figure 2b, it may also be necessary to extend the capillary inlet (18, 19). Nitrogen flow does not seem to be necessary for ion transmission, although nitrogen may be used to provide a dry sample region. The ions desorbed from the sample are drawn into the inlet capillary, assisted by applying high voltage on the sample probe or plate. The advantage of this geometry is that an x - y sample stage can be used to accommodate numerous samples.

Transmission geometries. Danell and Glish described an AP MALDI probe made from a glass capillary that uses a coaxial optical fiber to illuminate the sample from behind with a Nd:YAG laser operating in the third harmonic (Figure 3a) (16). They improved the source's performance by adding a heated coil to the glass transfer capillary, which assists in desolvating the ions desorbed by the AP MALDI source (20). They reported that heating the source resulted in mostly singly charged ions, elimination of cluster ions, improved reproducibility, and a detection limit lowered by 3 orders of magnitude.

Callahan and co-workers have also described an AP MALDI source with transmission geometry (Figure 3b) (17, 18). In a typical MALDI experiment, the laser irradiates the sample on the front side of an opaque surface, in a configuration known as reflection geometry. Transparent materials with either conducting or insulating properties were evaluated in these experiments; however, these properties seemed to have little effect on the ion source's performance. The sample slides were placed between two metal plates with holes through the center in order to apply high voltage and illuminate the sample plate from behind with laser light. A nitrogen laser focused onto the sample slide desorbed and ionized the analyte.

Because Callahan had previously constructed an AP MALDI with reflection geometry (17, 18), they were able to directly compare the two configurations. The transmission geometry required higher laser powers (150–190 μ J/pulse) than the reflection geometry (25 μ J/pulse) and produced signal intensities ~ 1 order of magnitude less than the reflection geometry. However, the spectra obtained from the transmission geometry lacked the analyte–matrix clusters observed in the reflection geometry.

In 2001, Mass Technologies introduced the first commercial AP MALDI source, which incorporated a 337-nm nitrogen laser, a fiber-optic system for transmitting the laser beam to the sample, an 8 × 8 sample chip, a CCD camera for sample visualization, and a computer-controlled *x*-*y* sample stage. A modified version of this source that is equipped with a UV (Nd:YAG at 355 nm) and IR (Er:YAG at 2940 nm) laser system is currently being used in our laboratory (Figure 4). The optical components of the source were modified to accommodate the 355- and 2940-nm laser outputs, and analyses are being done using both liquid and solid matrixes.

Mass spectra

AP MALDI produces results that are, in most respects, the same as those from vacuum MALDI. However, there are additional advantages, including interchangeability with ESI, the use of ion traps for MS/MS experiments, and the possibility of liquid matrixes. Thus, it is conceivable that AP MALDI will ultimately be the preferred MALDI method, at least for molecules falling within the mass range that can be transmitted by quadrupole and hexapole ion guides. For these samples, the oaTOF and ion trap mass analyzers offer the possibility of obtaining mass spectra in which resolution is unaffected by the initial conditions of the ionization process.

There is also some evidence that AP MALDI produces molecular ions with lower internal kinetic energies than those produced by vacuum MALDI. In one of their first applications of AP MALDI on a quadrupole-TOF mass spectrometer, Burlingame and co-workers obtained mass spectra of several phosphorylated peptides (conotoxins) from the venom of the marine gastropod *Conus pennaceus* (21). They observed that the mass spectra obtained by vacuum MALDI in the positive ion mode generally produced only dephosphorylated ions, whereas those from atmospheric pressure MALDI produced molecular ions of intact phosphorylated species. From this, they concluded that "the internal energy deposition during ionization/desorption by MALDI carried out at AP is modulated by collisional damping, which preserves a significant fraction of pseudomolecular ions intact for acceleration into the oaTOF analyzer" (21).

The tendency for AP MALDI to form cooler molecular ions can also result in clustering of molecular species with matrix molecules (Figure 5a). Adduction of one to four α -cyano-4-hydroxycinnamic acid molecules (m/z 1386.1, 1575.3, 1763.1, and 1953.7) occurs for the very basic peptide VRKRTLRL (m/z 1197.7) but not for gramicidin (m/z 1141.5). Figure 5b shows how an increase in the interoctapole potential on an AP MALDI ion trap by 50 V can promote declustering of the molecular species (15).

The ion trap mass spectrometer in our laboratory provides the opportunity to directly compare the performance of AP MALDI and ESI under the same instrumental conditions (Figure 2). Figures 5c and 5d compare the MS/MS spectra of the singly charged molecular ions of the phosphorylated peptide Ac-RRLIEDAE(pY)AARG-NH₂ obtained by AP MALDI and ESI. Both show a predominant loss of

98 mass units along with several sequence ions. The loss of 98 mass units corresponds to neutral H₃PO₄ and is commonly observed for phosphoserine and phosphothreonine peptides but less frequently for phosphotyrosine peptides. Because of the structure of the phosphotyrosine side chain, it is possible that the loss of 98 mass units corresponds to sequential loss of HPO₃ and H₂O. Recent work with this instrument has shown that the loss of 98 mass units is observed in both AP MALDI and ESI spectra when the amino acid sequence in the vicinity of the phosphorylation site contains a basic arginine or lysine residue (22). The peptide AALIEDAE(pY)AAAG, for example, does not show a loss of 98 mass units.

The stability of MALDI ions formed under atmospheric conditions may also make it possible to observe the same noncovalent complexes by AP MALDI, as in ES, but more easily than by conventional vacuum MALDI. Figure 5e shows the mass spectrum of a complex between the basic peptide dynorphin and the acidic peptide minigastrin (23).

Although a relatively new technique, AP MALDI is already solving structural problems in biology. Keough et al. used a commercial AP MALDI source on an LCQ mass spectrometer for analyzing sulfonic acid-derivatized tryptic peptides (24). Creaser and co-workers constructed an AP MALDI ion source with a fiber-optic interface for analyzing oligosaccharides (25). A recent report by Laiko et al. described using water and other non-denaturing liquid matrixes for IR (2940 nm) AP MALDI, which could find widespread application for characterizing biological systems (26).

Intermediate pressure methods

Standing and co-workers have developed intermediate-pressure MALDI sources using a quadrupole collisional damping interface on a tandem quadrupole/orthogonal injection TOF (QqoaTOF)

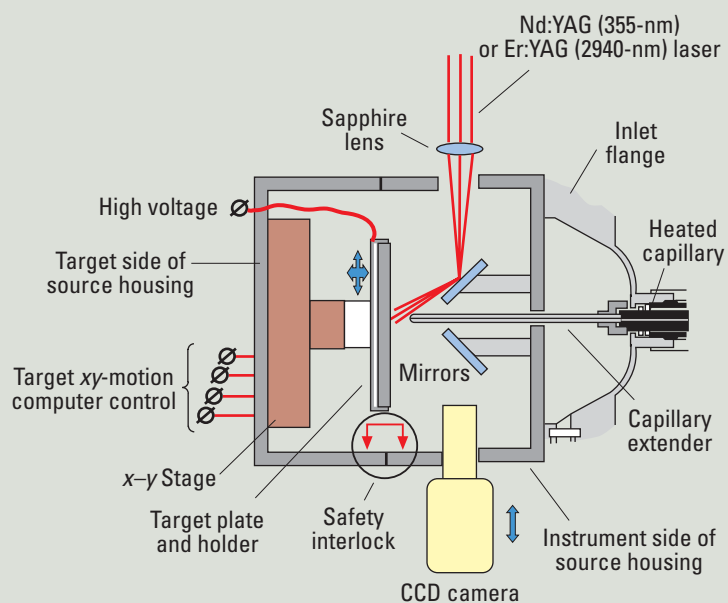


FIGURE 4. Modified commercial AP MALDI ion source used in our laboratory.

mass spectrometer (27, 28). Their work suggests that ionization at pressures near 1 Torr significantly decreases metastable fragmentation of MALDI ions. Baldwin et al. described a MALDI ion source operating at 8 mTorr for a QqoaTOF (29). In these configurations, low-energy collisions cool the energetic MALDI ions before they enter the first quadrupole, resulting in a quasi-continuous ion beam.

O'Connor and Costello introduced an intermediate-pressure MALDI source for an FT ion cyclotron resonance (ICR) mass spectrometer that uses a pulsed valve to introduce collision gas to the MALDI target region for collisional stabilization of MALDI ions (30). Baykut and co-workers have developed a MALDI source for an FTICR in which the MALDI target is located 1 mm from the entrance of the hexapole ion guide (31). The MALDI ions are collisionally cooled by pulsing gas into the area directly surrounding the MALDI target. The ions are trapped in the hexapole before injection into the ICR cell. Hexapole ion trapping allows for the accumulation of multiple

laser shots before mass spectral analysis, thus increasing the method's sensitivity.

Although many features of these intermediate-pressure methods are similar to those of AP MALDI, there is a fundamental instrumental difference: In intermediate-pressure methods, ionization is still carried out in a vacuum and thereby requires pumps, sample interlocks, and other equipment. In AP MALDI, large sample stages can be accommodated in an external ionization region and integrated with sample-handling systems and robotics. Thus, AP MALDI should provide a unique opportunity for the high-throughput methods that are being developed for genomic and proteomic screening.

Ion focusing

The current configuration of the AP MALDI source involves ion transfer through a heated capillary into an intermediate-pressure region in which ions are sampled by a skimmer into an octopole. This method of ion transport from AP to vacuum is

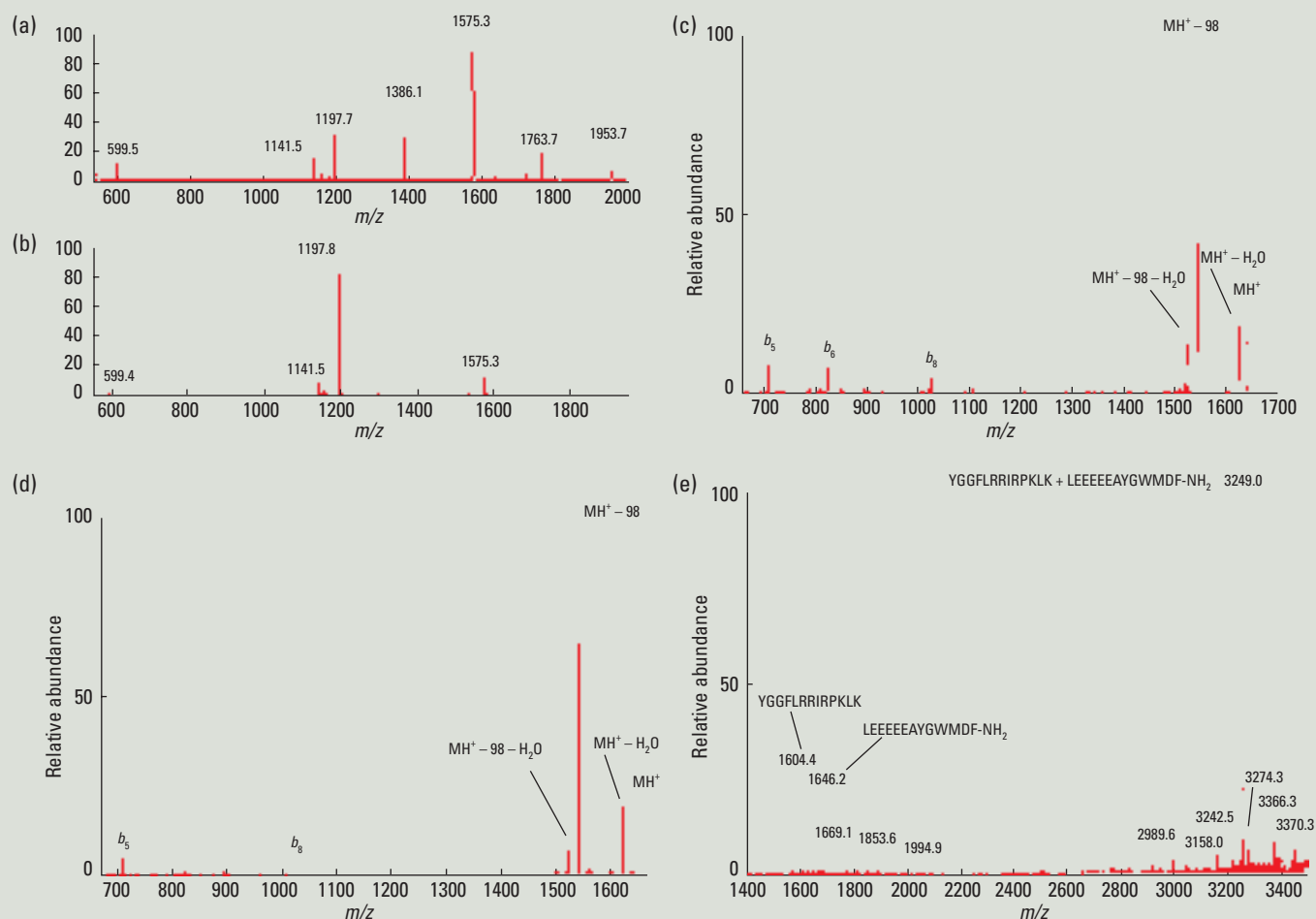


FIGURE 5. Peptides by ESI and AP MALDI ion trap MS.

(a) AP MALDI spectrum of a mixture of gramicidin and the peptide VRKRTLRLR, showing clustering of the protonated peptide with α -cyano-4-hydroxycinnamic acid matrix; (b) shows the declustering caused by raising the interelectrode voltage. (c) AP MALDI spectrum and (d) ESI spectrum of the phosphopeptide Ac-RRLIEDAE(pY)AARG-amide. (e) AP MALDI spectrum of dynorphin (YGGFLRRIRPKLK) and minigastrin (LEEEEEAYGWMDf-NH₂) in saturated 6-azo-2-thiothymine (ATT) matrix, showing a complex formed between the two peptides.

identical to that used in ESI and API, which use the capillary as part of the desolvation process, but its primary use in AP MALDI is for ion transport. The addition of a metal capillary extension to accommodate the geometry of the ion trap inlet region (Figure 4) does not seem to diminish sensitivity, which is consistent with the results obtained by Lin and Sunner for metal capillaries (32).

The ability to transmit MALDI ions formed at AP into the vacuum region more efficiently is critical at this point, because AP MALDI is significantly less sensitive than conventional MALDI. Fortunately, we believe that the ionization efficiency of AP MALDI is comparable with that of vacuum MALDI. However, improvements are needed in ion transport to the capillary, transmission through the capillary, expansion into intermediate-pressure regions (1–100 mTorr), and transfer and focusing into the high-vacuum region. The factors that affect space-charge-dominated sources like ESI and API are also critical to optimizing AP MALDI, as discussed theoretically by Busman et al. (33).

For the region between the inlet capillary and the ion guide (intermediate-pressure region), Smith and co-workers have done significant work developing electrodynamic ion funnels for better ion transmission from ESI sources into triple-quadrupole and FTICR mass spectrometers (34–37). The ion funnel consists of a series of cylindrical ring electrodes of decreasing internal diameter, with rf and dc electric fields applied to collisionally focus ions at elevated pressures. To improve the initial transport of ions (at AP) to the capillary, Schneider and Chen have developed the AP ion lens (38). The ion funnel and lens should find use on AP MALDI instruments in the near future.

Proteomics, biodetection, and diagnostics

There is a broad effort today to map the human proteome and to correlate expressed proteins with genomic data, medical histories, and specific diseases. Information obtained from proteomics research will aid in the identification of new drug tar-



The oaTOF and ion trap analyzers offer the possibility of obtaining spectra unaffected by the initial conditions of ionization.

gets, improvement of disease diagnostics capabilities, and determination of the most appropriate therapies for individual patients.

To address the needs of proteome research, an analytical technique must be capable of screening large numbers of samples quickly and reliably. For these reasons, MS is at the forefront of proteomics technologies. In particular, MALDI TOF chip-based techniques with 96- and 384-well plate formats have become the standard in MS proteomics research, and high-density chips are coming into increasing use. To improve the speed and efficiency of MS analyses, techniques must be developed to accommodate robotics and facilitate automation. Because AP MALDI places the ionization source external to the mass spectrometer, it may simplify the interface to robotics systems.

Sample pump-down time is eliminated, which improves analysis time, and the AP MALDI source can be equipped with UV and IR lasers for use with liquid and solid matrixes.

There is considerable interest in developing miniaturized and field-portable mass spectrometers for biological agent detection. We have collaborated with the Johns Hopkins University Applied Physics Laboratory to develop a field-portable TOF MS known as the TinyTOF, which

collects air samples and concentrates particles ranging in size from 0.5 to 10 μm onto a moving tape. The tape is sprayed with a MALDI matrix and introduced into the mass spectrometer, where it is ionized by a UV laser (39, 40). The resulting spectra are searched for biomarker patterns indicative of biological warfare agents. An AP MALDI system could provide an efficient method for such continuous monitoring.

Demirev et al. have demonstrated that protein databases derived from microorganism genomes can be used to positively identify peptide masses in mass spectra that are indicative of biological agents—an approach they have termed phyloproteomics (41). Field-portable instruments for monitoring infection or disease would also use proteomic approaches. So, there is not a significant difference in the instrumentation required for bio-agent detection and clinical diagnostics. In environmental, forensic, and clinical diagnostics laboratories, an instrument that

did not require the insertion of samples into a vacuum system would have a considerable advantage, particularly with unskilled operators. Samples would be read as in the ion-mobility-based detectors currently used at airport security checkpoints, but far more specific identification or diagnosis would be provided.

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