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Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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A novel ionization source for biological mass spectrometry is described that combines atmospheric pressure (AP) ionization and matrix-assisted laser desorption/ionization (MALDI). The transfer of the ions from the atmospheric pressure ionization region to the high vacuum is pneumatically assisted (PA) by a stream of nitrogen, hence the acronym PA-AP MALDI. PA-AP MALDI is readily interchangeable with electrospray ionization on an orthogonal acceleration time-of-flight (oaTOF) mass spectrometer. Sample preparation is identical to that for conventional vacuum MALDI and uses the same matrix compounds, such as α-cyano-4-hydroxycinnamic acid. The performance of this ion source on the oaTOF mass spectrometer is compared with that of conventional vacuum MALDI-TOF for the analysis of peptides. PA-AP MALDI can detect low femtomole amounts of peptides in mixtures with good signal-to-noise ratio and with less discrimination for the detection of individual peptides in a protein digest. Peptide ions produced by this method generally exhibit no metastable fragmentation, whereas an oligosaccharide ionized by PA-AP MALDI shows several structurally diagnostic fragment ions. Total sample consumption is higher for PA-AP MALDI than for vacuum MALDI, as the transfer of ions into the vacuum system is relatively inefficient. This ionization method is able to produce protonated molecular ions for small proteins such as insulin, but these tend to form clusters with the matrix material. Limitations of the oaTOF mass spectrometer for singly charged high-mass ions make it difficult to evaluate the ionization of larger proteins.

Over the past 30 years, the development of new ionization methods together with improvements in mass analysis has been the primary driving force behind the remarkable and dramatic growth of biological mass spectrometry. Ten years ago the invention of matrix-assisted laser desorption/ionization (MALDI)[1] and electrospray ionization (ESI)[2] established mass spectrometry as one of the most powerful tools for the detection, identification, and characterization of biopolymers such as peptides, proteins, and DNA. Both MALDI and ESI enable the production of intact heavy molecular ions from a condensed phase (solid for MALDI and liquid for ESI). While ESI involves ionization at atmospheric pressure followed by transfer to the vacuum system, MALDI ion sources operate under vacuum conditions. Accordingly, different types of mass spectrometers are commonly used for ESI MS and MALDI MS analysis.

A new atmospheric pressure ionization technique, atmospheric pressure matrix-assisted laser desorption/ionization (AP MALDI), has recently been developed in our laboratory.[3] AP MALDI has many features in common with conventional vacuum MALDI, including the nature of the matrix, the proportions of matrix and analyte, the sample preparation procedure, and the laser beam energy density at the target surface.[3] Both techniques are based on the process of pulsed laser beam desorption/ionization of a solid-state target material, resulting in production of gas-phase analyte molecular ions. For conventional MALDI, after laser desorption the target material density drops rapidly from the high value characteristic of the initial solid phase to a very low value corresponding to the high vacuum of the mass analyzer. By contrast, AP MALDI produces a more or less uniform ion cloud under atmospheric pressure conditions, which is a feature of all atmospheric pressure ionization methods. The initial processes of laser light absorption and the expansion of a gaseous plume containing the target material are most likely the same for both vacuum and AP MALDI, but in the case of AP MALDI, further specific atmospheric pressure processes such as thermalization of vibrationally excited ions and ion−ion or ion−molecule reactions may take place over an extended time period. A study of the physical and chemical processes underlying AP MALDI may contribute to an improved understanding of similar processes in vacuum MALDI, which remain the subject of considerable debate.[4] On the other hand, as a new atmospheric pressure ionization technique, AP MALDI offers considerable promise for analytical purposes. A preliminary investigation of this possibility is the primary focus of the current investigation.

EXPERIMENTAL SECTION

For the detection of ions produced by MALDI at atmospheric pressure, a Mariner orthogonal acceleration time-of-flight (oaTOF) mass spectrometer (PE Biosystems, Framingham, MA) was

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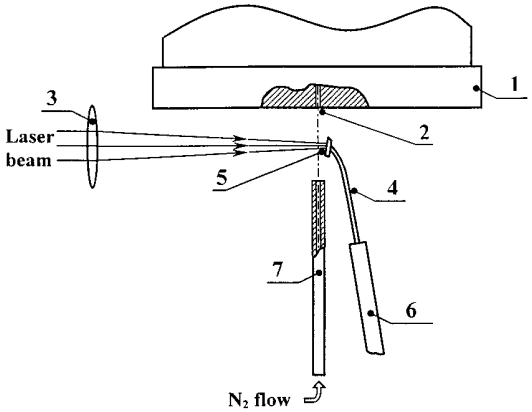

Figure 1. Schematic view of the PA-AP MALDI source. 1, Atmospheric pressure interface of Mariner oaTOF mass spectrometer; 2, inlet nozzle to MS instrument; 3, quartz lens; 4, replaceable target tip; 5, stainless steel MALDI target plate; 6, target holder; 7, stainless steel capillary gas nozzle.

equipped with a home-built AP MALDI source. Basically, any type of mass spectrometer with an atmospheric pressure interface could be used for this purpose, but the extended mass scale and high sensitivity of the oaTOF mass spectrometer make this a suitable choice for the analysis of biopolymers. The pneumatically assisted ESI source of the Mariner mass spectrometer was replaced with an AP MALDI source external to the mass analyzer. Several different variants were constructed and tested, the best of which from the point of view of sensitivity and stability is represented schematically in Figure 1. No further modifications were necessary to the Mariner instrument, to the normal operating conditions of nozzle voltage 125−175 V and temperature of 150 °C, or to the recording/processing software. For routine operation, the AP MALDI source is readily interchangeable with ESI; the replacement procedure is simple and takes only a few minutes.

Ultraviolet radiation at 337.1 nm from a pulsed nitrogen laser (model VSL-337ND, Laser Science Inc., Franklin, MA) was focused by a quartz lens with focal length of 150 mm onto a small replaceable stainless steel target plate, approximately 1.5 mm × 2.5 mm in size, situated 1−4 mm from the inlet to the atmospheric pressure interface of the mass spectrometer. The standard ESI needle potential of 1−5 kV was applied between the target electrode tip and the Mariner inlet nozzle. The electric field assisted the charge separation and ion transport processes in the atmospheric pressure region between the target electrode tip and the nozzle. To prevent corona discharge or electrical sparking, the replaceable target tips had no sharp edges. A standard MALDI sample preparation routine was used; a droplet of 1.5 μL of 1:1 v/v matrix/analyte solution was deposited on the target plate to cover the target surface completely and allowed to dry in 20−30 min. For all peptide/protein spectra illustrated, the matrix was α-cyano-4-hydroxycinnamic acid purchased as a standard solution (Hewlett-Packard, Palo Alto, CA). The peptide mixture was supplied by Bio-Rad (Hercules, CA). The oligosaccharide difucosyllacto-*N*-tetraose (Oxford Glycosystems, Wakefield, MA) was ionized from a 2,5-dihydrobenzoic acid (DHB) matrix (Aldrich). Neither ion beam collimation nor attenuation was used. The lens-to-target distance was adjusted to vary the size of the laser spot at the target to optimize the analyte ion current. The ideal laser spot was found to illuminate an area of 0.7−1.6 mm^2 with an energy density of 260−750 μJ/mm^2 per pulse, at a repetition rate of 20 Hz. No synchronization of laser pulses with the operation of the mass spectrometer was attempted.

An important addition to the ion source proved to be a stainless steel capillary gas nozzle (1.55 mm o.d., 0.5 mm i.d.), through which a stream of dry nitrogen was delivered at 0.4−0.8 L/min. This carried ions produced at the target surface toward the inlet orifice of the mass spectrometer. Although the ion source could operate without auxiliary gas flow, the sensitivity and stability of the pneumatically assisted-AP MALDI (PA-AP MALDI) version as represented in Figure 1 were considerably enhanced. This source was designed to facilitate the adjustment of the laser beam direction, beam focus, target electrode, and gas nozzle positions with respect to the inlet orifice, all of which played a significant role in ion current optimization.

PA-AP MALDI mass spectra were recorded using the Mariner instrument in the spectrum accumulation mode. After the data acquisition was started, the laser was switched on, and the target tip position was scanned slowly in the $x-y$ plane to expose the total target spot area to the laser radiation. The acquisition was stopped when the majority of the target material had been ablated by the laser beam and there was little or no further increase in the ion counts recorded. This process typically took 2−4 min, during which time up to 10^6 ion counts were accumulated to produce a spectrum. The noise level of the Mariner instrument (number of counts when the laser was off but all voltages were on) was 4−5 counts/s, or 500−1200 counts over the total acquisition time. The consumption of the entire analyte during the recording of a spectrum by PA-AP MALDI (which is also the case for electrospray[2]) is in sharp contrast to that of conventional vacuum MALDI,[1] where only a fraction of a single matrix/analyte microcrystal may be consumed from several hundred or even several thousand crystals on a typical target. The ionization of the total amount of analyte material deposited on the PA-AP MALDI target tip was necessary to compensate for the low efficiency of transfer of the ions from atmospheric pressure into the vacuum system.[5]

For a comparison of AP MALDI spectra with conventional vacuum MALDI, a Voyager Elite TOF mass spectrometer was employed in delayed extraction, reflectron mode. The sample preparation routine was the same as that described above for PA-AP MALDI, except that only 1 μL of analyte/matrix solution was deposited on the target plate for each sample.

RESULTS AND DISCUSSION

Sensitivity of Detection. The sensitivity for ion production and detection for analytical applications is a primary consideration for any ion source; thus, obtaining high sensitivity for the PA-AP MALDI source was a major objective of this work. This depended strongly on the geometry of the target tip and its position with respect to the inlet orifice, the laser beam energy density at the target surface, the applied voltage, the nitrogen gas flow rate, and the gas nozzle position. Optimization of all these parameters proved to be a complex and time-consuming problem; thus, the sensitivity level reported below is not necessarily the ultimate achievable. It is likely that this could be improved by further careful optimization of the source design and the experimental parameters.

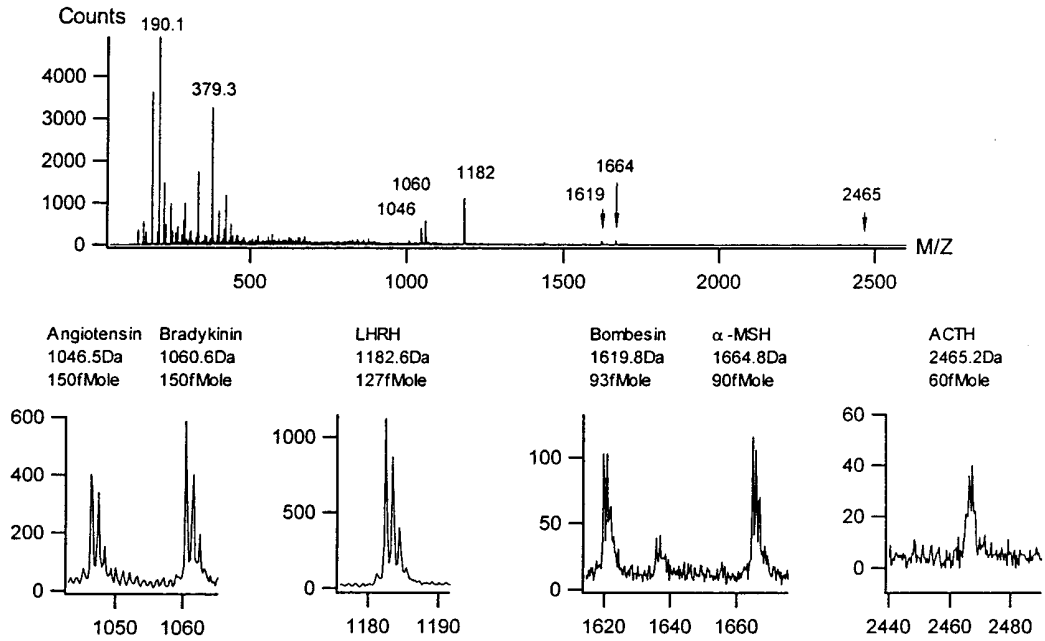

Figure 2. PA-AP MALDI spectrum of the mixture of six peptides, obtained by drying 1.5 μL of a 1:1 mixture of matrix (α-cyano-4-hydroxycinnamic acid, HP matrix solution) and analyte solution (0.22 ng/μL of each peptide) on the surface of the target electrode.

To evaluate the sensitivity of the PA-AP MALDI source for peptide analysis, we employed a standard solution of six peptides with molecular masses in the range of 1−2.5 kDa. This solution, which is commonly used in our laboratory for the adjustment and calibration of conventional vacuum MALDI instruments, was diluted to contain 0.2 ng/μL of angiotensin (M_r = 1046.5 Da), bradykinin (M_r = 1066.6 Da), LH−RH (M_r = 1182.6 Da), bombesin (M_r = 1619.8 Da), α-MSH (M_r = 1664.2 Da), and ACTH (M_r = 2465.2 Da), i.e., 80−200 fmol/μL. The PA-AP MALDI spectrum in Figure 2 demonstrates the sensitivity level achieved for 0.75 μL of this solution, containing 60−150 fmol per component. The strongest peaks in the low-mass region of this spectrum at m/z 190.2 and 379.3 are the protonated matrix monomer and dimer, which are also commonly observed in conventional MALDI spectra. The expansion of each peptide peak confirms that all are clearly discernible; even the weakest peak for 60 fmol of ACTH shows a recognizable isotope pattern with a signal-to-noise ratio >10.

The Softness of PA-AP MALDI Compared with That of Vacuum MALDI. The ability of an ionization method to produce low internal energy molecular ions with minimal fragmentation is of particular importance for the analysis of biochemical samples. The spectrum in Figure 2 demonstrates that PA-AP MALDI is a soft ionization technique, comparable with ESI and conventional vacuum MALDI. In fact, in contrast to vacuum MALDI, no fragment ions were recorded for PA-AP MALDI of these peptides, even at elevated laser beam energy densities. This specific feature of the new ion source may be explained as due to rapid thermalization of the ions by collision with ambient gas before fragmentation can occur, whereas in vacuum MALDI, excited ions can dissociate unimolecularly in the low pressure of the rapidly expanding "plume" to produce prompt fragments, or later during acceleration to give metastable ions.

Further confirmation of the softness of PA-AP MALDI ionization came from a comparison with conventional MALDI for ionization of a peptide containing oxidized methionine, Ser-Arg-Pro-Ala-MetO−His-Phe-NH_2 (Figure 3). The S−C bond in oxidized methionine (MetO) is labile, and a 64-Da loss (SO_2) is common for such peptides. Spectrum A in Figure 3, from conventional vacuum MALDI-TOF, gave a well-resolved molecular ion at m/z 860.8 and a poorly resolved broader metastable peak of apparent $m/z \approx$ 799, which could be confusing as the mass difference is less than the "correct" value of 64 Da by ~2 Da. Slow fragmentation of the vibrationally excited molecular ion during acceleration causes peak broadening and an apparent mass shift. Traces B and C of Figure 3 represent PA-AP MALDI mass spectra using an in-source fragmentation voltage (nozzle−skimmer potential difference) of 175 or 220 V, respectively. The lower voltage gave virtually no fragmentation, whereas the higher voltage gave a well-

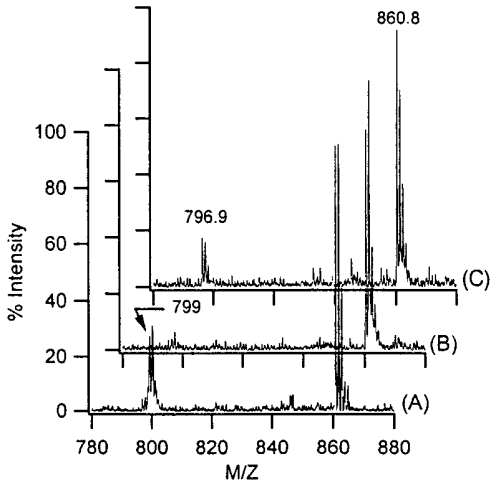

Figure 3. MALDI spectra of a synthetic peptide, Ser-Arg-Pro-Ala-MetO−His-Phe-NH_2, containing oxidized methionine (MetO). (A) Vacuum MALDI: Voyager Elite instrument, delayed extraction, reflectron mode. (B) PA-AP MALDI: 175-V nozzle−skimmer voltage. (C) PA-AP MALDI: 220-V nozzle−skimmer voltage.

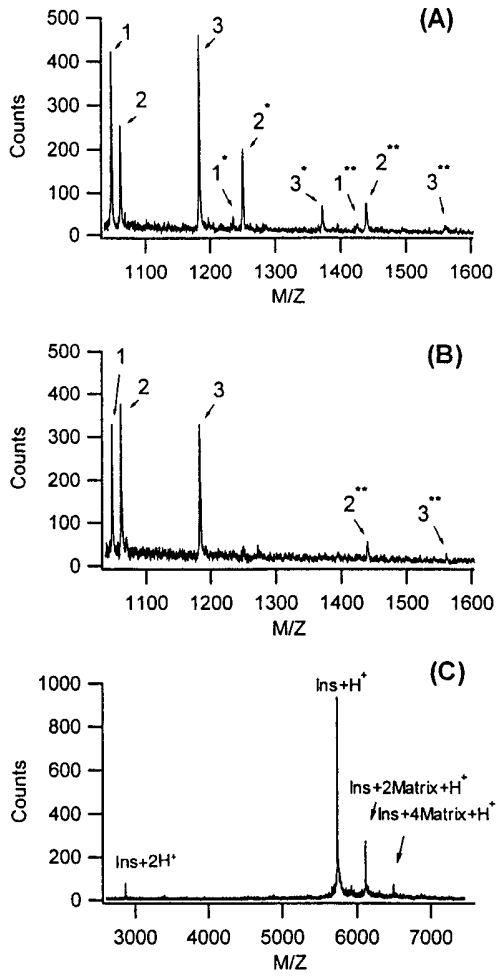

Figure 4. (A,B) PA-AP MALDI spectra showing analyte−matrix cluster formation for a mixture of angiotensin (1), bradykinin (2), and LH−RH (3). Designations: *, A + M + H^+; **, A + 2M + H^+, where A is analyte and M is matrix. Laser energy density (A) 267 and (B) 420 μJ/mm^2. (C) PA-AP MALDI spectrum of bovine insulin (M_r = 5733.5, Sigma).

resolved peak of m/z 796.9, confirming the loss of 64 Da. This demonstrates that, despite the softness of the ionization, PA-AP MALDI combined with the oaTOF mass spectrometer enables the use of controllable fragmentation to provide structural information. Alternatively, coupling this ion source with a tandem mass spectrometer such as a triple quadrupole or a QqTOF hybrid would give more comprehensive sequence data. The softness of this ionization method allowed the ionization of intact sulfated conotoxin peptides that are normally prone to decomposition.[5]

Analyte−Matrix Cluster Ion Production. Having identified PA-AP MALDI as a softer ionization technique than vacuum MALDI, we anticipated this could be particularly useful for the production of intact protein molecular ions, but the softness of the ionization gave rise to an abnormally high level of analyte−matrix cluster ions. Figure 4A represents a region of the PA-AP MALDI spectrum of the same peptide mixture used to generate Figure 2, but recorded with a lower laser beam energy density of 267 μJ/mm^2, close to the ionization threshold below which point the recorded total ion current dropped drastically. In addition to the intense protonated angiotensin, bradykinin, and LH−RH peptide peaks, we observed analyte + matrix + H^+ (*) and analyte + 2(matrix) + H^+ (**) peaks which were significantly stronger for bradykinin than for either angiotensin or LH−RH. Thus, these cluster ions are dependent on the chemical nature of the analyte. Figure 4B, recorded at 420 μJ/mm^2, demonstrates that the clusters were reduced at higher laser energies.

Cluster ions were stronger for heavier analyte molecules such as proteins, distributing the ion current over a series of peaks, thereby decreasing the sensitivity. Figure 4C shows the PA-AP MALDI spectrum obtained from 7.5 pmol of bovine insulin (Sigma, M_r = 5733.5), recorded with a laser beam energy density of 330 μJ/mm^2 and a nozzle−skimmer voltage of 350 V. The nitrogen flow rate and target tip position were adjusted to achieve the best sensitivity and to suppress the cluster ions as much as possible, conditions that gave an excellent signal-to-noise ratio. A notable feature was the nearly complete suppression of odd-numbered matrix clusters rather than even-numbered. No conditions were identified that would completely remove the residual clusters with two and four matrix molecules. Such analyte−matrix cluster ion formation is partly responsible for the lower sensitivity of AP MALDI for heavy molecules of M_r > 6 kDa. Although some additional information may be extracted from the study of relative cluster intensities, their presence may also complicate the interpretation. Possible solutions to this problem include the use of other matrixes already employed in vacuum MALDI and a search for new specific matrixes for AP MALDI of heavy molecules.

AP MALDI for Multicomponent Peptide Mixture Analysis. The comprehensive analysis of a mixture such as a protein digest and the identification of modified peptides without prior separation remains a challenge for mass spectrometry and for MALDI MS in particular. To investigate the applicability of PA-AP MALDI to multicomponent mixture analysis, a spectrum was recorded for 1.5 pmol of a tryptic digest of bovine fetuin, a well-characterized glycoprotein of 341 amino acids.[6] In previous studies of this tryptic digest in our laboratory by HPLC-ESIMS, 42 peptides and glycopeptides in the mass range 456−8327 Da were successfully identified.[7] PA-AP MALDI gave peaks corresponding to 29 of these components in the mass range 500−6340 Da, including six glycopeptides of M_r > 4 kDa. An equivalent analysis by vacuum MALDI identified only 16 peptides with an upper mass limit of 3 kDa, which included no glycopeptides. Figure 5 shows the 1.9−4.3 kDa region of both spectra, in which eight components were clearly identified by PA-AP MALDI but only three by conventional MALDI. The prominent ions of m/z 2026.1, 2568.5, and 3508.9 in the PA-AP MALDI are probably also weakly present in the vacuum MALDI spectrum, but the inferior signal-to-noise ratio prevented a precise monoisotopic mass assignment. There are several possible reasons for the better data quality in the case of PA-AP MALDI: the entire sample is consumed, thus the signal is averaged over the very inhomogeneous target surface; this is a softer ionization source so heavier peptides/glycopeptides are less likely to fragment; furthermore, any desorbed neutral analyte

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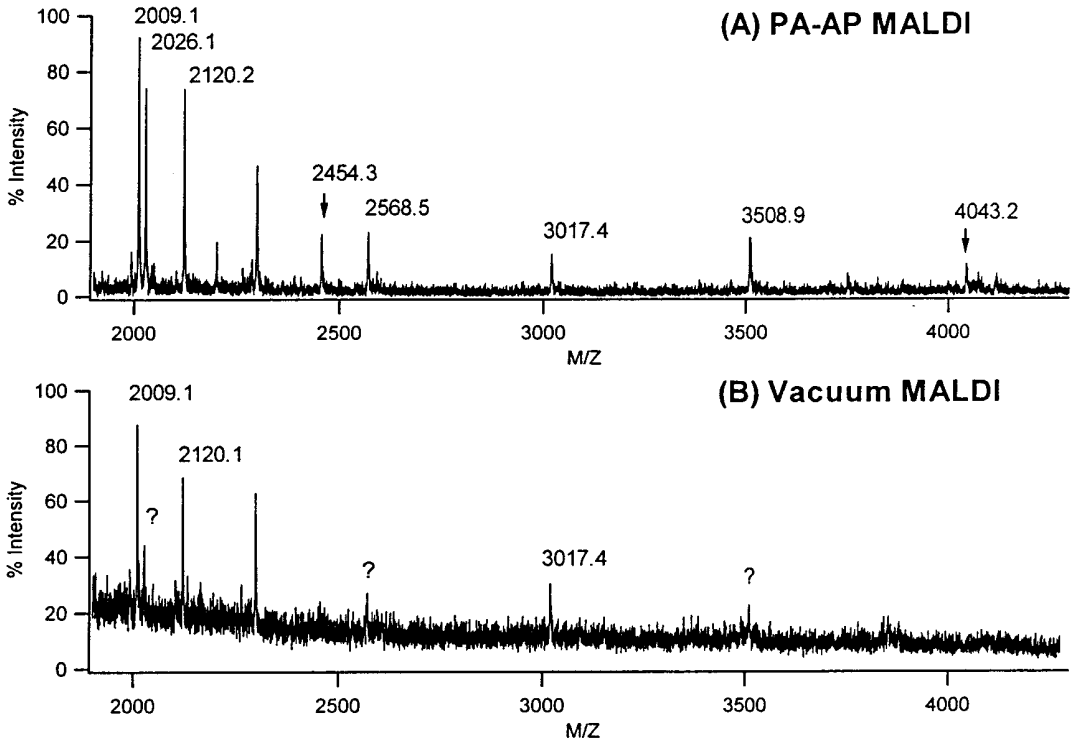

Figure 5. Partial mass spectra for tryptic digest of bovine fetuin. (A) PA-AP MALDI spectrum from 1.5 pmol deposition, showing eight peaks previously identified from LC/MS data.[6] (B) Vacuum MALDI spectrum from Voyager Elite instrument in delayed extraction reflectron mode for 1 pmol deposition, showing three peaks previously identified from LC/MS data.[6]

molecules are more likely to become protonated in the atmospheric pressure region of the PA-AP MALDI source.

PA-AP MALDI Mass Spectrum of an Oligosaccharide. Although the examples presented above all deal with the analysis of peptides and proteins, this technique is also suited to other classes of polar involatile compounds. As an example, Figure 6 presents the PA-AP MALDI spectrum of a branched hexasaccharide, difucosyllacto-*N*-tetraose ionized from DHB, which is generally considered to be a "cool" matrix for standard MALDI; i.e., it imparts relatively little internal energy to the analyte ions. As is quite common for oligosaccharides in MALDI, the molecule readily bound a sodium ion to give the sodiated molecular ion at m/z 1022.3. This compound showed a surprisingly high level of fragmentation, although formation of the strong B_3 ion of m/z 658.2 by cleavage adjacent to the *N*-acetylhexosamine would be anticipated as a favored process. Further loss of fucose from this ion gave the more intense peak at m/z 512.2; thus, it was possible to break multiple bonds. This compound failed to give a satisfactory conventional vacuum UV−MALDI spectrum. It did yield a spectrum from a succinic acid matrix in negative ion mode using an Er−YAG infrared laser operating at 2.94 μm, but of much inferior signal-to-noise ratio compared with that obtained with PA-AP MALDI.

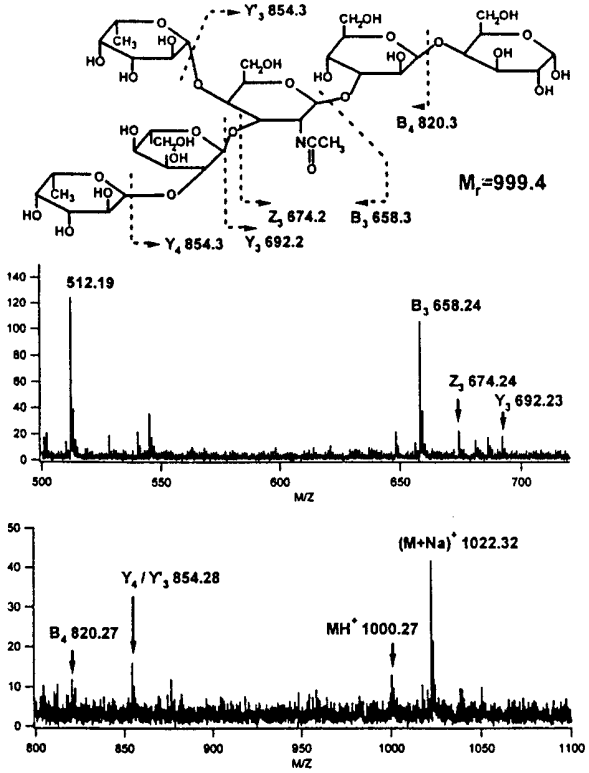

Figure 6. PA-AP MALDI spectrum of the branched oligosaccharide difucosyllacto-*N*-tetraose ionized from DHB matrix. The fragment ion nomenclature is from Domon and Costello.[14]

CONCLUSIONS

PA-AP MALDI is a new atmospheric pressure ionization technique, shown here to be applicable to the analysis of peptides, proteins, and oligosaccharides, which has much in common with conventional MALDI. Basically, the same sample preparation methods may be used successfully for both techniques, and PA-AP MALDI spectra are rather similar to those from conventional vacuum MALDI. Thus, the question is, what are the possible

advantages of PA-AP MALDI from the point of view of its analytical applications?

One advantage is the possibility of using the same instrument for both electrospray and MALDI spectral measurements. Unlike vacuum MALDI, AP MALDI at a laser frequency of 20 Hz gives an almost continuous ion current, due to the thermalization of the ions, similar to ESI. This affords more flexibility in the nature of the mass analysis technique and makes AP-MALDI readily compatible with an oaTOF mass spectrometer, the duty cycle of which provides high sensitivity.[8] The source described here, when interfaced with the Mariner oaTOF mass spectrometer, could detect <100 fmol of peptide of M_r up to 2.4 kDa with a signal-to-noise ratio >10, a level of sensitivity comparable to that of a typical ESI source. The comparison is less straightforward for conventional MALDI, in which only a tiny fraction of target material is consumed. Although typically 0.1−1 pmol of analyte mixed with matrix may be deposited per MALDI target, most of this material remains intact after the spectrum is recorded; thus, the true sensitivity of MALDI can be greatly enhanced by optimized sampling methods. A further advantage of PA-AP MALDI interfaced with the oaTOF mass spectrometer is the linearity of mass calibration, allowing highly accurate mass assignments. By comparison, the introduction of the delayed extraction technique in conventional MALDI-TOF enabled an increase in the resolution,[9−13] but at a cost of dependence of calibration on the delay time and on initial ion velocities. In turn, the initial ion velocities are dependent on ion mass; therefore, the most precise mass assignment is possible only in the vicinity of calibration reference peaks.

An indication of the fundamental, inherent sensitivity of the PA-AP MALDI ionization method was obtained by employing supplementary corona discharge to produce what might be described as an AP MALDI/APCI source. A platinum wire of 0.2 mm diameter was inserted into the gas nozzle, and a potential of 5−5.5 kV was applied, which produced a corona discharge in the AP region between the wire and the AP MALDI target. Reactive ions produced by such a discharge will participate in ion/molecule reactions to protonate analytes containing basic sites, such as peptides. In practice, the intensities of the matrix ion signals were increased 20−40 times, whereas peptide ion peaks were largely unaffected by the discharge. This suggests that the majority of the analyte molecules are ionized directly by the laser desorption process without the benefit of corona discharge. Consequently, the major limitations for sensitivity of AP MALDI relate to the efficiency with which ions can be transferred through the mass spectrometer interface and into the high vacuum of the mass analyzer. A more powerful pumping system such as is now available on several commercial mass spectrometers would allow larger nozzle and skimmer orifices; thus, ion transmission of API interfaces could be improved substantially in the next generation of orthogonal TOF instruments.

PA-AP MALDI also benefits from its softness, a feature that is of primary importance for molecular ion production for polar biomolecules containing weak molecular bonds and that could potentially make this method useful for the analysis of larger proteins. Unfortunately, the accelerating voltage of the Mariner instrument employed here is limited to ∼5 kV, and the sensitivity of the channel plate detector drops significantly for ions of high m/z with kinetic energies of only 5 keV. To further investigate the possibility of producing heavy protein molecular ions under API conditions, a higher acceleration voltage and/or additional ion postacceleration would be required. Also, the quadrupole lens in the interface discriminates against ions of higher m/z; thus, a redesigned interface would be required to optimize the transmission of heavier ions.

The almost complete consumption of the analyte proved to be necessary for PA-AP MALDI analysis to compensate for the ion losses in the API interface of the mass spectrometer. This feature may be considered a disadvantage of the technique, as analysis of the same target cannot be repeated several times. On the other hand, extensive sample consumption lends itself to automated spectral measurement, in which the laser beam could be rastered across the entire sample. A substantial number of microcrystals of the inhomogeneous target material would contribute to a final spectrum that was relatively independent of sample preparation, unlike the case in conventional MALDI, which relies more on identifying "sweet spots" on the target surface, making the development of an automated protocol more challenging. With the dramatic growth in sample throughput required for the new science of proteomics, automation of sample handling and analysis in mass spectrometry is both essential and inevitable.

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