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Detection and analysis of polymerase chain reaction products by mass spectrometry

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ABSTRACT

While molecular weight determination of proteins by matrix-assisted laser desorption/ionization (MALDI) has been quite successful, the measurement of deoxyribonucleic acid polymers in a size range useful for fine-structure genome analysis (50-1,000 bases; ca. 15,000-300,000 amu) has proven more difficult, due to decreasing sensitivity and resolution at the higher molecular weights. Tailored cleanup techniques and the use of delayed extraction time-of-flight mass spectrometry are approaches that offer considerable promise for improved MALDI analysis of DNA.

Keywords: mass spectrometry, MALDI, laser desorption, DNA

1. INTRODUCTION

The accurate measurement of the molecular weight of deoxyribonucleic acid (DNA) oligomers is of great importance in many disciplines ranging from molecular biology and medical diagnostics to agriculture and forensic sciences. Widely-used assays encode biological information, such as the presence of a normal or altered gene, identity of a criminal or victim, or the sequence of a targeted DNA region, in the sizes of the resulting DNA oligomer products (oligonucleotides). Such assays include chemical or enzymatic sequencing[1] and the polymerase chain reaction (PCR)[2]. The current workhorse method for size measurement of the oligonucleotides produced in these assays is gel electrophoresis. In gel electrophoresis, whether performed in a slab or capillary configuration, oligonucleotides of differing size are separated due to differences in migration rate through a gel medium under the influence of an applied electric field. Gel electrophoretic separation and detection of oligonucleotides has several drawbacks. The process is generally fairly slow and labor intensive, although developments in the use of multiple capillaries or "microchip" electrophoresis offer some improvement in this regard. To allow detection of the bands on the gel corresponding to different sizes, the oligonucleotides must generally be fluorescently or radioactively labeled prior to the separation, or stained afterward. The "size" inferred by migration rate through a gel can be a complex function of several factors, including the actual base sequence of the oligonucleotide and solution conditions, so that the accurate molecular weight determination by gel electrophoresis requires some care. Despite these shortcomings, however, electrophoresis provides single-base resolution of oligonucleotides containing up to several hundred bases or base pairs, and is therefore a widely applied "readout" method for assays such as nucleic acid sequencing or PCR analysis.

Shortly after the development of matrix-assisted laser desorption/ionization (MALDI) combined with time-of-flight mass spectrometry (TOF) as an analytical technique for large biomolecules,[3] several laboratories began to assess the utility of this and related laser desorption ionization techniques for biomolecule analyses traditionally performed by gel electrophoresis. The rationale behind using mass spectrometry as an alternative to gel electrophoresis was the apparent vast improvement in analysis speed that could be realized. While a slab gel electrophoresis run with autoradiographic detection can take more than one day, a single-laser-shot time-of-flight mass spectrum can be obtained in less than 1 millisecond, a factor of some 10^8! However, practical considerations such as loading the sample into the mass spectrometer, locating a productive "spot" on the sample with the laser, averaging mass spectra from several laser shots together, and data transfer and analysis, dictate a typical MALDI analysis time of at least several minutes, bringing the time closer to 10^2. One might argue that the time savings possible with mass spectrometry almost disappears when one considers performing many electrophoresis runs in parallel or the use of faster emerging electrophoretic methods.

Despite what may be a rather shaky argument for greater analysis speed using mass spectrometry, a number of researchers are actively pursuing the goal of DNA detection by MALDI-TOF techniques because of other potential advantages over electrophoretic or hybridization techniques. The accuracy of molecular weight determination using mass spectrometry, which directly measures the mass to charge ratio (m/z) of gas-phase ions, should be substantially greater than electrophoretic techniques. While current "read lengths" (related to the largest size of DNA that can be detected with

single-base resolution) are considerably shorter for mass spectrometry (about 100 bases) than for gel electrophoresis (several hundred bases), this figure of merit, which is important for analysis of DNA sequencing reactions, is continually improving for MALDI-TOF. Simultaneously, there is a growth in application of MALDI-TOF mass spectrometry to other assays besides sequencing that yield DNA products of smaller size and in fewer numbers than sequencing, so that single-base resolution of sizes up to hundreds of bases is not required. For instance, we have previously described the analysis of PCR products from the human DNA.[4] In this work, a PCR assay was designed to produce a product flanking a region of the cystic fibrosis transmembrane conductance regulator gene. For a normal copy of this gene, the resulting PCR product contains 75 bases, while a 3-base deletion (ΔF508) that causes cystic fibrosis results in a 72-base product. These products are smaller than those resulting from a "standard" PCR assay for the ΔF508 mutation, which was at that time beyond the upper mass limit which we could reliably detect by MALDI-TOF. We have also demonstrated the detection of bacterial PCR products from the *Legionella* genus, containing either 108 bases or 168 bases.[5] Other laboratories have reported detection of PCR products and other oligonucleotides containing in excess of 600 bases.[6]

This paper describes recent and ongoing efforts to overcome some of the obstacles to more routine and robust application of MALDI-TOF to analysis of PCR products and other information-bearing nucleic acid molecules. Methods for purifying nucleic acid samples are described, as is the application of delayed extraction TOF mass spectrometry to analysis of short oligonucleotides.

2. EXPERIMENTAL

MALDI sample preparation involves mixing a solution containing the DNA be analyzed with a solution containing the matrix compound. For the results described here, the matrix used was 3-hydroxypicolinic acid (HPA).[7] The mixture of matrix and DNA is applied to a metal solids probe, the solvent is allowed to evaporate, and the sample is transferred to the source region of (typically) a time-of-flight mass spectrometer. A pulsed laser is used to irradiate the sample. The matrix compound, typically a small organic aromatic acid, is chosen to absorb light strongly at the laser wavelength, so that the laser pulse rapidly heats and desorbs the matrix, which entrains and ionizes the DNA to produce intact, gas-phase ions that can be mass analyzed. DNA samples were either synthesized or produced by the polymerase chain reaction.[4] To reduce the effects of alkali metal cation adduction to the DNA, cleanup procedures, such as ethanol precipitation from ammonium acetate solution and the use of molecular weight cutoff filters, were employed.[4] Cation exchange beads in the ammonium form were also added to the final matrix/DNA solution on the probe tip to remove residual alkali metal ions.[8]

Although other types of mass spectrometers can be used to analyze MALDI-generated ions, the most commonly used is the time-of-flight mass analyzer (TOF). In this instrument, ions are accelerated by an electric field to constant energy. The ions then enter a field-free drift region where they are separated on the basis of m/z due to their different velocities. Two distinct linear TOF configurations were used to obtain the results described here, depending on whether the accelerating field applied in the ion source was continuous or pulsed. For continuous extraction, a relatively long (2 meter) field-free region was used, with extraction potentials up to ±20 kilovolts allowing analysis of either positive or negative ions.[9] For pulsed extraction experiments, a shorter field-free region of approximately 25 cm was used with an extraction potential around +3 kilovolts. The length of the shorter field-free region for pulsed extraction measurements was determined based on the correlation of an ion's initial position and velocity in the MALDI.[10] For both TOF configurations, a nitrogen laser operating at 337 nm provides the desorption pulse. Ions are detected at the terminus of the field-free drift region using a modified dual microchannel plate detector and a digital oscilloscope. Data are transferred to a personal computer for analysis.

3. RESULTS AND DISCUSSION

3.1 Sample treatment and cleanup procedures

Oligonucleotide samples resulting from processes such as DNA sequencing or PCR contain significant concentrations of reagents that severely interfere with analysis of the products by MALDI. The major interfering species are salts, buffers, and unreacted primers (short oligonucleotides that recognize complementary sequences on target DNA and are incorporated into the larger oligonucleotide product of the assay). Salts are deleterious because the presence of different cations (Na, K, etc.) that pair with the multiple anionic sites on the DNA phosphate backbone can lead to a distribution of molecular weights for the DNA, resulting in a broadened peak in the MALDI spectrum. Excess amounts of salts may also

affect the crystallization of the matrix compound, leading to poor sensitivity. The presence of excess primers in a MALDI sample can also suppress the signal observed for the larger DNA analyte.[5] Cleanup procedures to remove salts and primers involve chromatographic methods, dialysis, molecular weight cutoff filters, and ethanol precipitation.[4-6,8]

As an example of the importance of removing alkali metal cations from nucleic acid samples, Figure 1 shows the effect on MALDI-TOF resolution and sensitivity of a rapid cleanup procedure based on microcentrifuge-tube format molecular weight cutoff filters. The spectra shown are for phenylalanine-specific transfer RNA (tRNA, molecular mass ca. 24.9 kDa, from brewers yeast; Boehringer-Mannheim). Two μL of a 110 μ**M** aqueous solution of the tRNA was diluted in 100 μL of either pure water, 0.1 **M** aqueous ammonium citrate solution, or 10^{-4} **M** NaCl. Each solution was centrifuged through a separate 10 kDa molecular weight cutoff filter (Rainin). A 100 μL aliquot of deionized water was then added to each filter tube for a second centrifugation. The tRNA retained in each tube was then mixed with HPA, and MALDI-TOF spectra were obtained (Figure 1, b-d) using continuous extraction conditions on the 2-meter TOF. Additionally, an aliquot from the 0.1 M ammonium citrate/tRNA mixture was analyzed by MALDI-TOF before the second centrifugation step with pure water (Figure 1a). The spectra obtained from the tRNA initially diluted in water (Fig. 1c) and 10^{-4} **M** NaCl (Fig. 1d) are broader and flatter than that obtained from the ammonium acetate solution (Fig. 1b). Presumably, the large excess of ammonium ions helps to displace other counterions from the multiply-anionic sugar-phosphate backbone of the t-RNA, resulting in a more nearly "single-salt" form of the analyte. However, if the excess ammonium is not removed by a second water wash, the overall signal level is suppressed (Fig. 1a). This suppression is not observed if a second wash is omitted from the water or 10^{-4} **M** NaCl solution of tRNA (not shown).

3.2 Resolution enhancement using delayed extraction

Maintaining a field-free state in the MALDI-TOF source region for a short, fixed interval before applying the accelerating field has been shown to improve dramatically the resolution.[10] Preliminary results presented below from a MALDI-TOF instrument with geometry optimized for pulsed or delayed extraction are promising. The design of this instrument has revealed the importance of some subtleties that are not as critical in high-voltage continuous extraction MALDI-TOF instruments.

Figure 2 shows SIMION[11] calculations of the potential gradients in the ion source for two slightly different geometries. The left-most electrode in these models represents the sample probe, from which the sample is desorbed. Along with this probe electrode, an intermediate grid, maintained at 268 volts, defines the first accelerating stage of the ion source. The intermediate grid is attached to one face of a thin (0.025") stainless steel plate; Figures 2a and 2b differ in the orientation direction of the grid-bearing face of this plate. This intermediate grid and a final grid, maintained at -1700 volts, define the second accelerating stage, and the final grid marks the beginning of the field-free drift region. (Positive ions travel from left to right in Figure 2.) For a short time after the laser fires, the sample probe electrode is maintained at the same potential as the intermediate grid, but is pulsed to +1309.5 volts following the delay to accelerate the ions toward the detector. The potential gradient contours are shown for the sample probe electrode at 1309.5 volts. In Figure 2a, the grid-bearing face of the intermediate grid mount faces the final grid. In this case, the potential gradient between the probe and intermediate grid along the central axis of the instrument varies from ca. 63 V/mm to ca. 67 V/mm. In contrast, Figure 2b shows that when the grid-bearing face of the intermediate grid mounting plate faces the probe electrode, the potential gradient between the probe and intermediate grid shows somewhat less variation. While this difference seems minor, Figure 3 demonstrates that this slight change in geometry has a significant effect on the predicted resolution. Figure 3a shows the total flight time after application of the accelerating pulse as a function of an ion's nascent velocity, which it acquires as a result of the laser desorption event. The "probe-facing" orientation of the intermediate grid (center trace) more closely approximates the behavior of an "ideal" delayed-extraction TOF experiment (upper trace) than does the "final grid-facing" orientation of the intermediate grid (lower trace). Figure 3b compares the ratio of average total flight time to the spread of flight times for different nascent velocities as a function of the time at which the accelerating voltage is applied to the probe electrode. The quantity $T/\Delta T$ is thus related to mass spectral resolution. For the "probe-facing" orientation of the intermediate grid, the optimum predicted $T/\Delta T$ is >3000, while for the opposite orientation of the intermediate grid, the predicted $T/\Delta T$ under optimum conditions is <1500. The better resolution predicted for the "probe-facing" orientation probably results from the fact this geometry provides better field uniformity between the probe and intermediate grid, where ions have not yet attained full acceleration.

The short configuration of our TOF instrument is based on the calculations described above. While the predicted

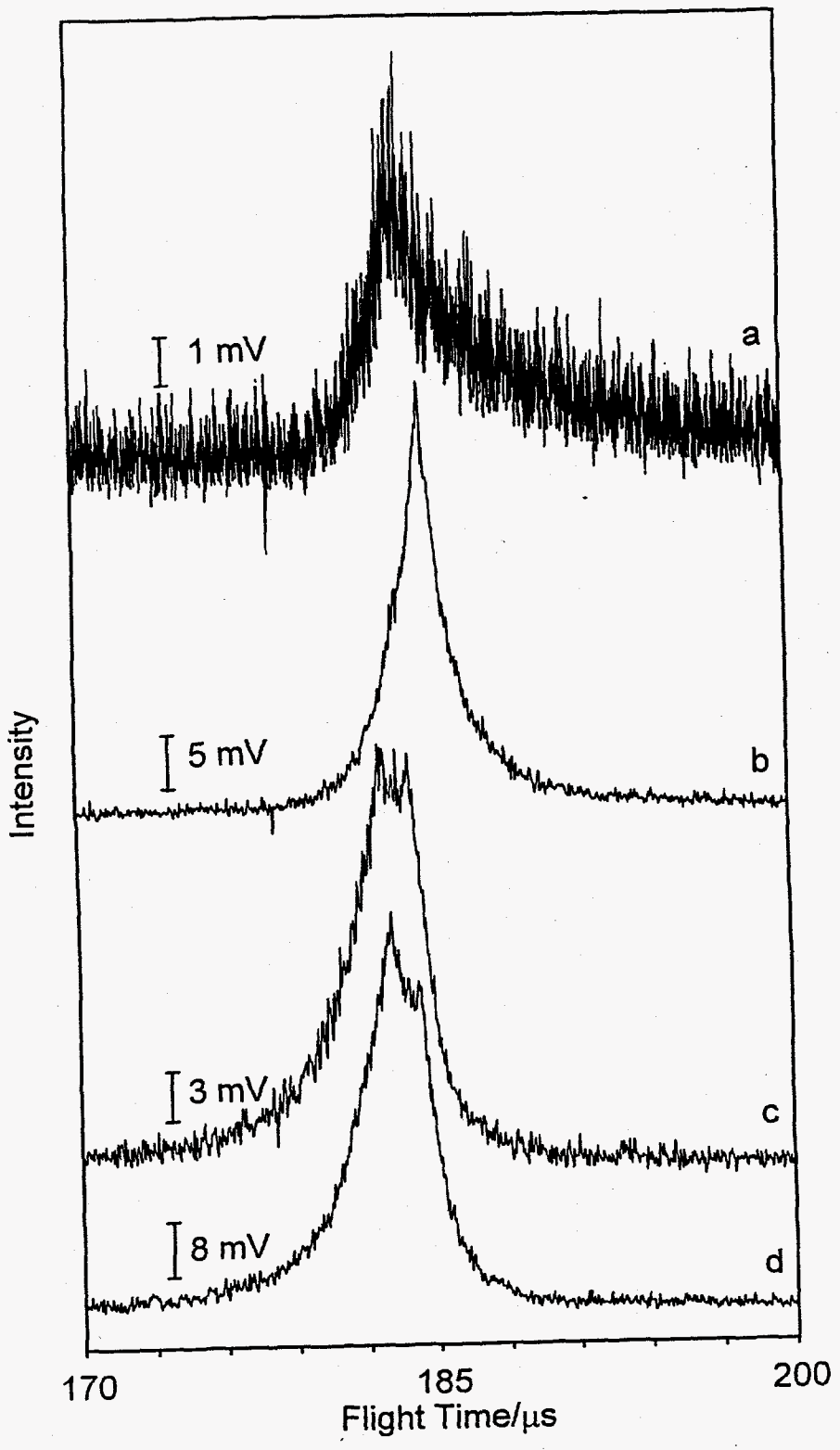

Figure 1. MALDI-TOF mass spectra of tRNA subjected to different sodium-removal steps. (a). 1.1 μ**M** tRNA in 0.1 **M** ammonium citrate solution, Single centrifugation step through 10 kDa MWCO filter. (b) Same as (a), with additional centrifugation step using pure water. (c). 1.1 μ**M** tRNA in pure water, subjected to two centrifugation steps through 10 kDA MWCO filter. (d) 1.1 μ**M** tRNA in 10^{-4} **M** NaCl, subjected to 1 centrifugation through 10 kDa MWCO filter, followed by second centrifugation through 10 kDa MWCO filter using pure water.

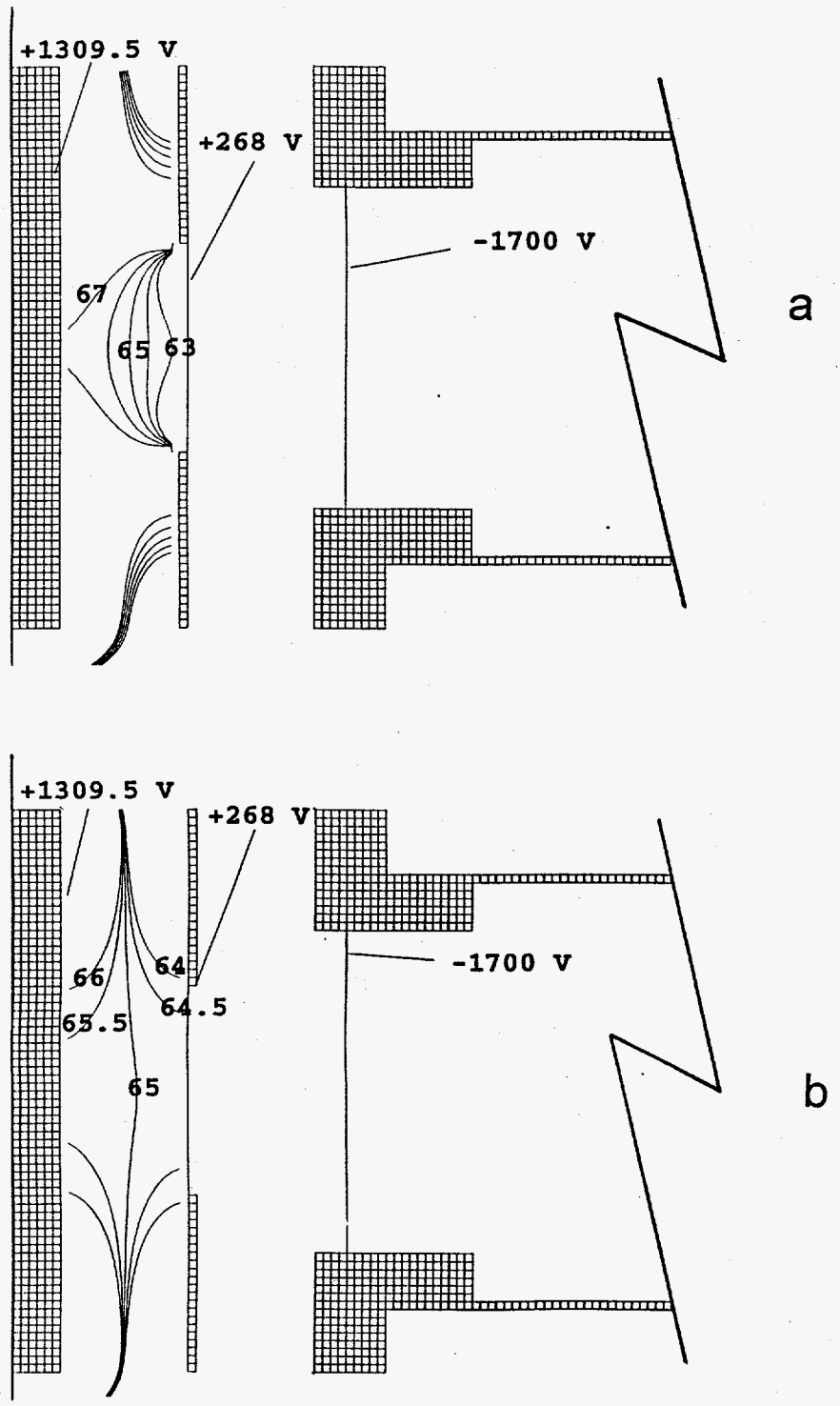

Figure 2. SIMION models of the source region of a short TOF instrument used for delayed extraction experiments. The potentials applied to the electrodes are indicated, as are potential gradient contours in V/mm. Each small square is 1 mm on a side. The thin vertical lines represent ion-transmitting grids, while adjacent squares represent solid electrodes.

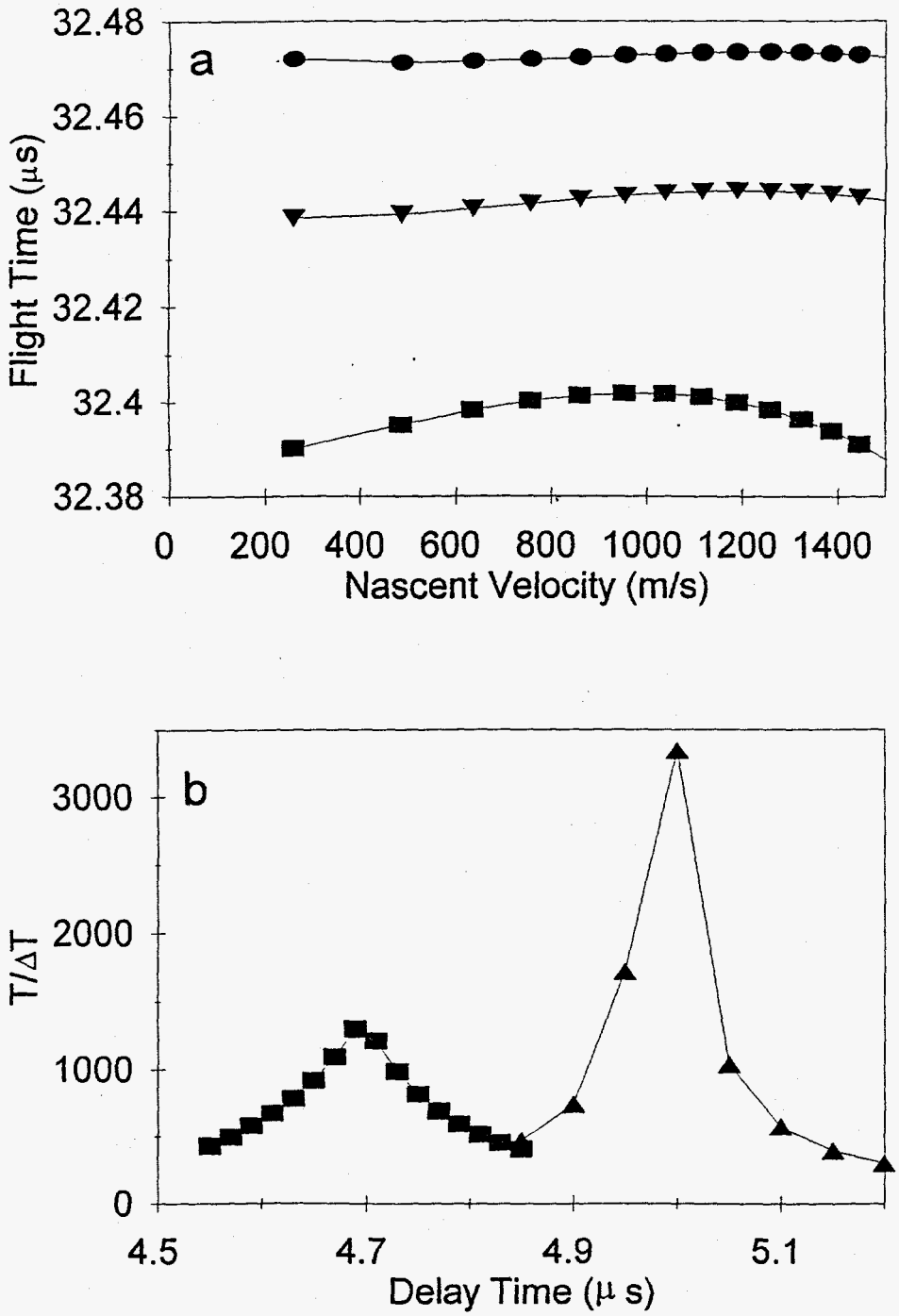

Figure 3. (a) Flight times as a function of n ascent velocity for MALDI-desorbed ions. Circles: "ideal" TOF instrument. Triangles: Model corresponding to Figure 2b. Squares: Model corresponding to Figure 2a.

(b) Average total flight time divided by flight time spread for different delay times before application of the accelerating field in the delayed extraction TOF. Squares: Model corresponding to Figure 2a. Triangles: Model corresponding to Figure 2b.

resolution has not yet been attained experimentally, preliminary results are encouraging. Figure 4 shows delayed-extraction positive-ion MALDI-TOF mass spectra of several small oligonucleotides ranging in size from a 20-mer to a 42-mer. With the relatively low total accelerating voltage used (ca. 3200 V), the signal-to-noise ratio drops off rather rapidly with increasing molecular weight. The resolution observed in these spectra, however, is ample to distinguish oligonucleotides differing in length by a single nucleotide, as shown by the spectrum of a mixture containing a 21-mer, 23-mer, 24-mer, and 29-mer.

Figure 5 compares the calculated isotopic distribution with the delayed-extraction MALDI-TOF spectrum of a DNA 20-mer, sequence 5'-GAC AGG AAA GAC ATT CTG GC-3', monoisotopic molecular mass 6172 Da. The major contribution to the mass spectral peak width is from the isotopic distribution of the compound. The full width at half maximum height (FWHM) of the calculated isotope distribution is some 3 Da, while the FWHM for the experimentally-observed mass spectral peak is ca. 7 Da, giving an effective resolution (m/Δm) of ~ 860. This spectrum, obtained with a total accelerating potential of 3200 V and the "short" TOF configuration, exhibits resolution comparable to or better than that obtained with the long field-free region, high-voltage TOF configuration used in previous continuous extraction experiments.

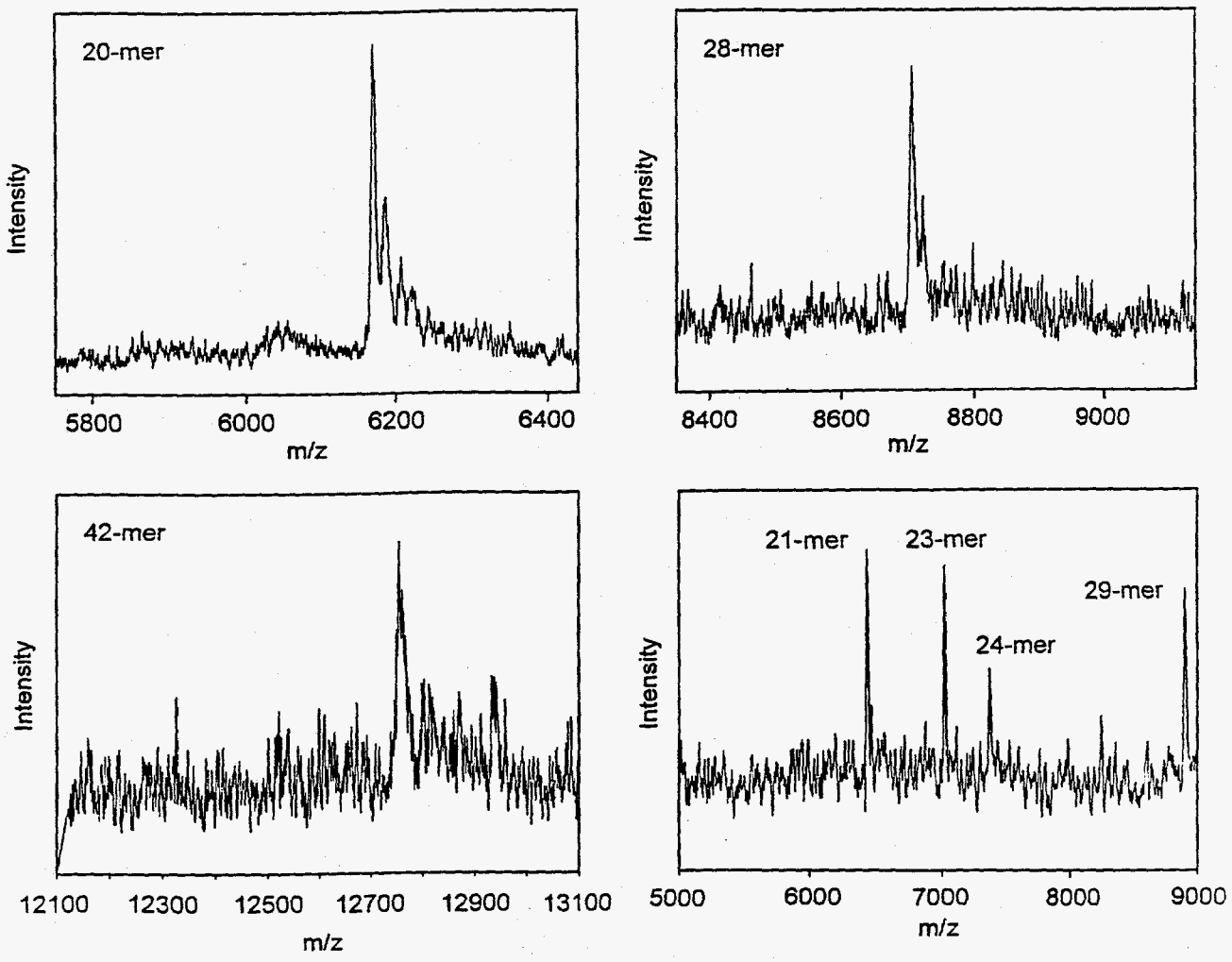

Figure 4. MALDI-TOF mass spectra, positive-ion mode, for several oligonucleotides.

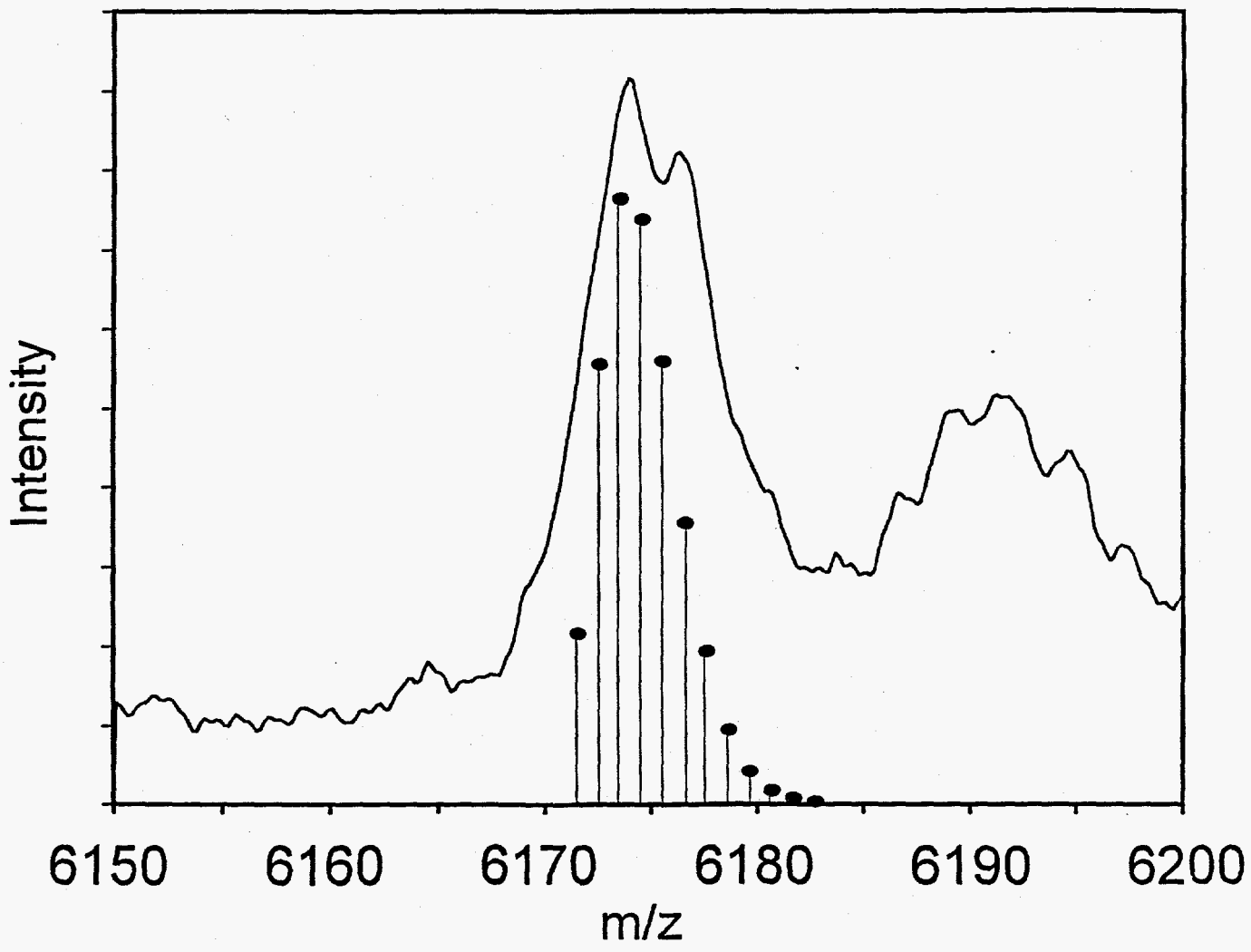

Figure 5. Comparison of observed MALDI-TOF delayed-extraction mass spectrum of a DNA 20-mer (solid line) with predicted isotope distribution for the 20-mer (stick spectrum). The second peak at ca. 6192 Da corresponds to an ammonium adduct of the 20-mer.

4. ACKNOWLEDGEMENTS

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