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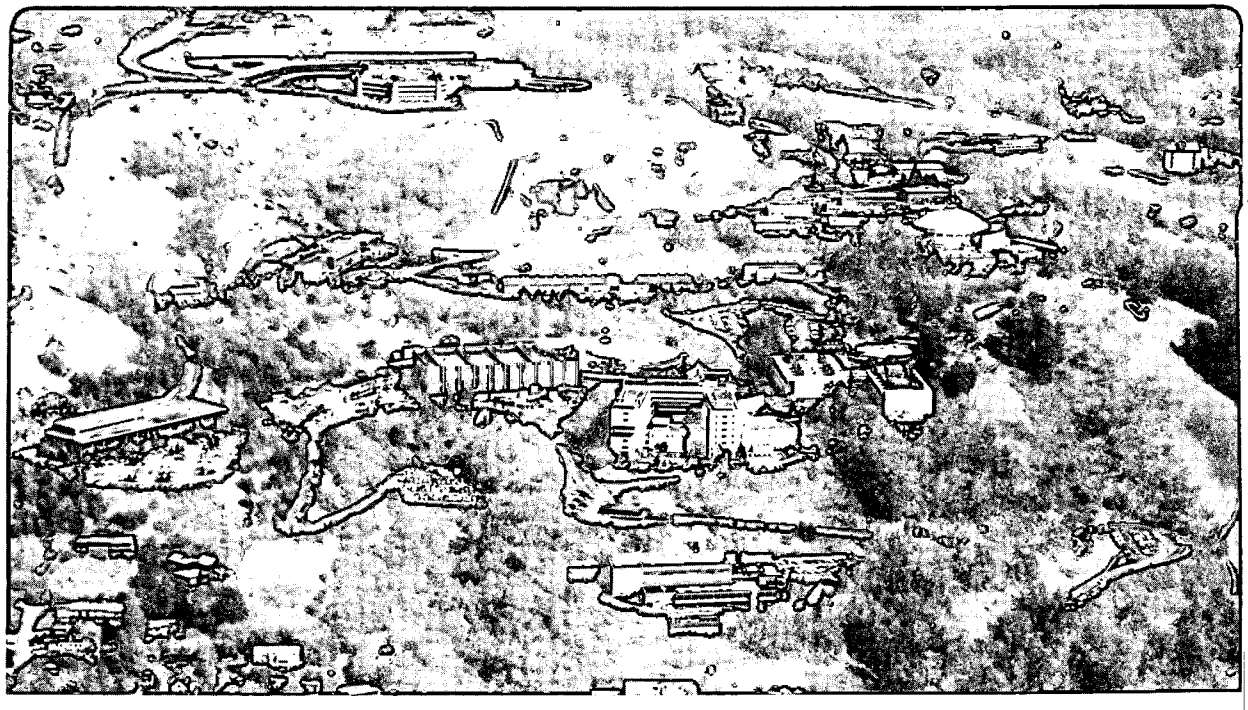

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"Identification of Denatured dsDNA by MALDI-TOF Mass Spectrometry"

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Identification of Denatured dsDNA by MALDI-TOF Mass Spectrometry

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Abstract

We created double-stranded DNA (dsDNA) by annealing synthetic oligonucleotides and used matrix assisted laser desorption (MALDI) time-of-flight (TOF) mass spectrometry (MS) to distinguish the difference in molecular weight of the complementary strands after denaturation. Refined sample preparation and deliberate rectilinear construction of a linear mass spectrometer produced a mass resolution exceeding 1000 for single-stranded-DNA (ssDNA). We present methodologies and demonstrate the potential for analyzing dsDNA by determining the mass of the complementary strands.

Key words: MALDI, mass spectrometry, oligonucleotide, DNA

Introduction

Time-of-flight techniques for ions theoretically span a limitless mass range as long as timing signals can be generated for the ion. The flash of the laser simultaneously produces the ion and a starting signal, and the collision of the ion onto a detector, such as a micro-channel plate, generates the stopping signal in matrix-assisted laser desorption/ionization time-of -flight mass spectrometry (MALDI-TOF-MS). TOF techniques continue to be attractive for determining the molecular weight of large molecules such as, but not limited to, oligonucleotides and DNA fragments. Current difficulties associated with the generation of unfragmented DNA ions limit the applicability of MALDI-TOF-MS technology to relatively small DNA molecules. Recent advances suggest that increasingly larger fragments of ssDNA and dsDNA will be determined by TOF techniques. The work of Schneider and Chait[1] showed that part of the problem for ionizing oligonucleotides relates to the ease with which certain adjacent nucleotides split apart. Wu[2] reported the identification of a matrix material, 3-hydroxypicolinic acid (3HPA), that increases the mass range of MALDI techniques for oligonucleotides to about 100 bases. Other matrices have been used to launch DNA by MALDI[3-5] but we prefer this one, to date. The addition of diammonium citrate (DAC) to this matrix, as reported by Pieles[6], also helps to extend the mass range and improves the mass resolution for oligonucleotides. Tang *et al.*[7-8] reported recently the mass determination of a 150-mer and a 500 bp fragment of DNA using a combination of this matrix and picolinic acid, but while many investigators use nitrogen lasers emitting 335 nm light to excite the 3HPA, they choose to use 266 nm illumination to excite the picolinic acid component.

We report, first, the construction of a linear MALDI-TOF mass spectrometer with which we achieved a resolution of 1000 (M/Δ m) for oligonucleotides, and secondly, the application of this mass spectrometer for detecting the molecular weight of each strand after short dsDNA molecules are denatured with heat. In the following text, DNA refers to oligonucleotides, ssDNA and dsDNA, but by itself, dsDNA emphasizes the analysis of denatured strands of complementary oligonucleotides.

Methods

We constructed a Wiley-McLaren[9] type of TOF mass spectrometer utilizing several additional features that enabled high resolution in a linear instrument. The physical positioning of the source plate, the sample holder and the detector were carefully aligned to be orthogonal. Although

orthogonality was not measured in the assembled instrument, parts were machined to tolerances to produce orthogonality better than 90 ± 0.3 degrees. The major deviation from orthogonality stemmed from small misalignments when flanges were welded onto vacuum tubing. Standard 4 inch diameter stainless-steel tubes with 6 inch Conflat flanges sealed with polymer gaskets enclosed the approximately 1 m ion flight path. A mechanically pumped vacuum (<1 mTorr) interlock provided a way to introduce samples into the vacuum chamber on the end of an electrically insulated rod (1.25 cm diameter, Nema-G) without introducing excessive gas loads during insertion of the sample probe. A 450 L/s turbomolecular pump (Varian, Lexington, MA) reduced chamber pressure to 10^{-7} Torr (Varian multigauge with cold cathode sensor) several minutes after the sample probe was slid through the vacuum interlock. A pulsed nitrogen laser (Laser Science, Inc., model VSL335ND) illuminated the sample at 45° with a 3 ns pulse of 335 nm light. The size of the illuminated spot was adjustable with a 250 cm focal length silica lens and was typically 0.12 X 0.25 mm. A translatable linear neutral density filter (Newport, Irvine, CA) adjusted the illumination intensity and a pivoting mirror in the laser beam deflected the illuminating spot radially across the sample. A pellicle reflected a small fraction of the beam into a photodiode (~1 ns rise time) for generating start signals. The sample holder and its supporting rod rotated continuously with a gear assembly and drive motor at about 0.3 revolutions/min. A 6 mm diameter stainless steel button was the sample holder that slid into a slightly larger hole in a 7.5 cm. diameter source plate. A spring finger mounted on the stationary source plate rubbed the rotating sample holder, thereby providing robust electrical contact. The sample holder from which ions were launched and the surrounding source plate were machined flat to <0.025 mm so that electric field lines across the surface of the sample and the surrounding plate were flat and did not cause the ions to be focused or defocused. This source plate was maintained at high voltage (0-30 kV d.c. power supply, Glassman High Voltage, Co., model EL30R01.5, 50 ppm regulation). Glassman modified this supply after purchase because the supply voltage regulator circuitry was not stable at the very low currents we require. The ion acceleration region consisted of a 1.27 cm space between the source plate and an electrically grounded plate on which a nickel screen (Buckabee-Mears, St. Paul, MN, 70 lines/in) stretched across a centered 0.80 cm diameter aperture. It was glued with silver paint onto the side of the ground plate that faced the source plate and care was taken to keep the screen flat and the glue as thin as possible. Positioned 5 cm downstream from the ground plate, a 0.18 mm diameter wire stretched axially in the flight path and served as an ion guide. The focusing wire was segmented; the first two-thirds of the 84 cm-long wire was set at ± 20 V to focus the ions (wire polarity opposite to ion polarity) and the final third was electrically isolated and optionally pulsed (Berkeley Nucleonics Co., 6040 Universal Pulse Generator, model 310H high voltage module (0-900 V, 0-20 msec pulses) to deflect matrix ions. Another 7 cm diameter plate, containing a 2.5 cm diameter aperture, covered by a nickel screen (see above), was positioned 2 cm from the end of the wire ion guide and electrically shielded the dual micro-channel plate ion detector from the pulsed segment of the wire ion guide. Located 2 cm downstream from the aperture, the microchannel plates defined the end of the ion flight path. A cylinder secured to the inside of the flight tube held the mounting brackets for the focus wire, the detector shield and 5 insulating posts which helped guide and support the microchannel plates. The microchannel plate assembly (TOF-2003 with 25 mm diameter detector-grade plates, Gallileo Electro-Optics, Sturbridge, MA) provided 50 ohm electrical connection to external amplifiers. The microchannel plates were electrically mounted to a 6 in diameter vacuum flange with a BNC connector supplied by the manufacturer, but were secured in place by the 5 insulating posts mentioned above. These posts supplied rigid support for several different detectors, including the microchannel plates we describe, and did not allow the detector assemblies to tilt nonperpendicularly. Thick (2 mm diameter) wires supplied high voltage (-1.8 kV to the front of the first microchannel plate and -0.1 kV to the back of the second plate) from SHV feedthroughs in the end cap. These thick wires provided a low inductance path and helped reduce ringing in the fast rise-time pulses. A preamplifier (LeCroy, Spring Valley, NY, model W101ATB, <2 ns rise-time) amplified the 2–5 ns wide microchannel plate pulses before they were digitized (LeCroy digital oscilloscope, model 7200, 500 MHz bandpass maximum, 2 GHz maximum digitization, 4 M-byte maximum memory

length). Mass spectra were summed and averaged with software resident in the LeCroy 7200. Depending on the application, an additional amplifier/discriminator (Mech-Tronics Nuclear) processed the signals before digitization, to improve sensitivity and stretch the pulses. Typical spectra were produced by digitizing at 400 MHz and averaging (n=50-5000 laser pulses) 1.35 V, 15 ns FWHM pulses. Tofware (Ilys Software, version 2.1K, Pittsburgh, PA) post-processed the LeCroy-generated data records.

Samples were prepared by evaporating mixtures of matrix and analyte with a gentle flow of N_2 or by the more recent procedure of Vorm *et al.*[10] Molecular ratios (Mr) of matrix/analyte indicate our use of the former technique. The complementary oligonucleotides were mixed in nearly equal molar concentrations (typically 2-10 X 10^{-4} M) in a microcentrifuge tube and allowed to anneal for several minutes. Then the tube was suspended in boiling water for 1 minute and an aliquot of the heat denatured dsDNA was quickly added to the matrix solution. The samples were typically prepared by evaporating, with blowing N_2, the mixture of 1 μL of 0.1 M matrix in 1:1 acetonitrile-water with 1 μL of 10^{-4} M oligonucleotide in water and 2 μL of 0.1 M aqueous diammonium citrate. The surface of the sample holder, roughened with 400 grit sandpaper, facilitated small crystal formation, along with the process of rubbing the surface during sample evaporation with the tip of a disposable micropipette. Sigma Chemical Co. (St. Louis, MO) supplied the 3-hydroxypicolinic acid (3HPA). We purchased acetone and acetonitrile from VWR Scientific (San Francisco, CA) and deionized water to > 18 M-ohms. The laser intensity in the MALDI mass spectrometer was adjusted to a level that was slightly greater than threshold for production of analyte ions, ranging between 30 and 80 mJ/cm^2. Not every laser pulse produced an analyte ion at the slightly greater than threshold intensity.

We either purchased (Genset, La Jolla, CA) or synthesized three pairs of complementary oligonucleotides with solid-phase-based phosphoramidite chemical methods recently automated into an 8 channel device.[11] Oligonucleotides were chosen to construct complementary strands of DNA. The oligonucleotides did not possess end trityl or 5' phosphate groups. We found that, in general, HPLC purification greatly diminished the concentration of Na^+ adducted components, but some preparations needed to be additionally purified. For this extra cleanup, we used ultrafiltration centrifuge cartridges (Micron-3, 3000 Da cut-off, Amicon, Beverly, MA) which further reduced Na^+ and K^+ contamination in the oligonucleotide solutions. Oligonucleotide solutions were centrifuged through these cartridges, and the residue retained on the ultrafiltration membrane was redissolved in water before analysis.

The base composition and molecular weights of the three oligonucleotide pairs we generated and the difference in molecular weight of the complementary strands are as follows:

823+: 5'-d(CTA ACT AGG GGC AAG TAC ATG C) -3'	22 bases	6768.4 Da	
823-: 3'-d(GAT TGA TCC CCG TTC ATG TAC G) -5'	22 bases	6701.4 Da	Δ = 67 Da
869+: 5'-d(CAC AGG AAG GAT ACA ATG TGT G) -3'	22 bases	6832.4 Da	
869-: 3'-d(GTG TCC TTC CTA TGT TAC ACA C) -5'	22 bases	6636.4 Da	Δ = 196 Da
886+: 5'-d(GAG TGA GTA TCT GGC TTC ATC C) -3'	22 bases	6741.4 Da	
886-: 3'-d(CTC ACT GAT AGA CCG AAG TAG G)-5'	22 bases	6728.4 Da	Δ = 13 Da

Results and Discussion

We attempted to determine if heating the solutions of complementary oligonucleotides would efficiently denature the dsDNA and if the MALDI sample preparation procedure would prevent the complementary strands from reannealing. To see each strand as a separate mass peak in the mass spectrum, the separation of the 886 +/- pair, requires an analytical resolution ((M/Δ m) where M=molecular weight at center of the peak and ΔM=FWHM of peak) of about 1000. The required resolution to see the separate strands of the other two complementary pairs is less constrained. We have recently observed a resolution greater than 1000 for HPLC purified oligonucleotides with MALDI-TOF-MS, and therefore reasoned that this type of mass difference measurement might be possible for short strands of dsDNA. Our experience, along with that discussed by other investigators[12], suggested that Na^+ cationization of the oligonucleotides could preclude the measurement for the 886+/- pair, but that the analysis of the 823+/- and 869+/- pairs would not be jeopardized by minor Na^+ adduction because of their larger mass differences. We added an oligonucleotide standard to the 823+/- pair for mass calibration purposes even though we knew the molecular weight of the synthesized oligonucleotides. This addition provided us with a 3-point mass calibration curve because the dimer peak of the calibrant $(2M+H)^+$ was evident. The molecular ion of the matrix comprised the third calibration point. We used the 3-point mass calibration curve to calibrate subsequently recorded spectra. The mass values labeling the peaks in the mass spectra shown in the attached figures resulted from this calibration.

Figure 1 shows a MALDI-TOF mass spectrum of a 22-base long oligonucleotide analyzed by the sample preparation procedure we described above. The addition of DAC, as described above, helped to overcome adduct interference when used cooperatively with the spin cartridge purification step. Too much DAC, such as the use of 0.5 M solution instead of 0.1 M, prevents appropriate crystal growth and leads to an oil-like residue from which ions are difficult to desorb. The resolution for the oligonucleotide was 1000, demonstrating that it is feasible to analyze oligonucleotides with small differences in mass, providing the solutions are purified. Results such as this suggest that oligonucleotides differing in molecular weight by as little as 10–15 Da could be analyzed as long as the Na^+ and K^+ adducts are minimized.

The sample preparation procedure we followed did not produce mass signals for dsDNA. After the strands of dsDNA were separated by heat denaturation, mass peaks for the ssDNA strands became evident. Three mass spectra are shown in Figs. 2–4. The spectrum for the 823+/- pair was obtained on the first try. The oligonucleotides in the remaining two pairs contained more Na^+ and K^+ and were noticeably more difficult to separate. We do not imply that Na^+ contamination or adduction influenced interstrand bonding, although we do not discount this idea. Two passes through an ultrafiltration spin cartridge adequately reduced the Na^+ contamination in the solution containing the 869+/- pair (Fig. 3); however, these complementary oligonucleotides reannealed during the first two attempts to prepare a sample and the spectrum showed only evidence of the oligonucleotide present in excess concentration. We initially heated the oligonucleotides in boiling water for one minute and then added an aliquot of this solution, along with DAC and matrix, onto a room temperature sample holder. Not until we added the mixture onto a sample holder heated to 100° C were we able to prevent the oligonucleotides from annealing during the time the solvent evaporated. Even after two passes through an ultrafiltration cartridge, K adducts are present in concentrations exceeding the pure molecule (Fig. 3).

Mass peaks corresponding to each complementary strand in the 886+/- pair are shown in Fig. 4 but bona fide mass assignments are compromised for the 886+ strand because it lies near the mono-Na adduct mass position of the 886- oligonucleotide. Try as we might, we could not reduce sodium contamination in this sample enough to verify the presence of the 886+ strand in the sample, even though the 886- strand is adequately resolved from other components in the sample. It was also

necessary to prepare this sample on a heated sample probe to prevent the denatured strands from annealing during solvent evaporation. It took three tries to adequately purify the oligonucleotide solution and two more attempts to denature this sample before the spectrum in Fig. 4 was recorded.

We can now routinely purify and denature solutions containing complementary strands less than about 30 base-pairs long. We have not yet tried to perform this analysis on longer strands. We continue to observe that complementary pairs of oligonucleotides show varying tenacity to reanneal following denaturation by heat. This observation appears to be in accord with the so-called melting temperature (T_m), a measure of the bonding strength between complementary strands. A higher temperature is required to denature G-C rich strands from each other because G and C share three hydrogen bonds, whereas A and T share two hydrogen bonds.[13] We do not claim to show T_m dependent on base composition for the complementary strands studied here, but offer the concept of T_m as a reason to expect that some complementary strands might be more difficult to denature than others.

We demonstrated the capability to mass analyze denatured dsDNA as large as 22 base pairs (~6900 Da) with a mass resolution (M/Δ m) as high as 1000. The mass spectrometric resolution attributes to a careful construction of a linear instrument in which the physical components are held orthogonally, the digitization of microchannel plate pulses with a 500 MHz bandpass, 1 GHz signal processor, and the art of sample crystallization to produce small crystals. The results presented demonstrate one way to analyze short strands of dsDNA. Because the MALDI approach does not generate mass signals readily for dsDNA, the preservation of denatured DNA in the MALDI matrix allows the double-stranded material to be detected indirectly. The extension of this type of analysis to longer complementary strands would provide a way to replace gel-electrophoresis with mass spectrometry for the analysis of PCR reaction product mixtures. It is not yet possible to detect several-hundred base-long denatured strands of dsDNA routinely, but the capacity to detect 150-mers reported by Tang *et al.*[14] suggests this may be feasible in the future.

Acknowledgments/Disclaimer

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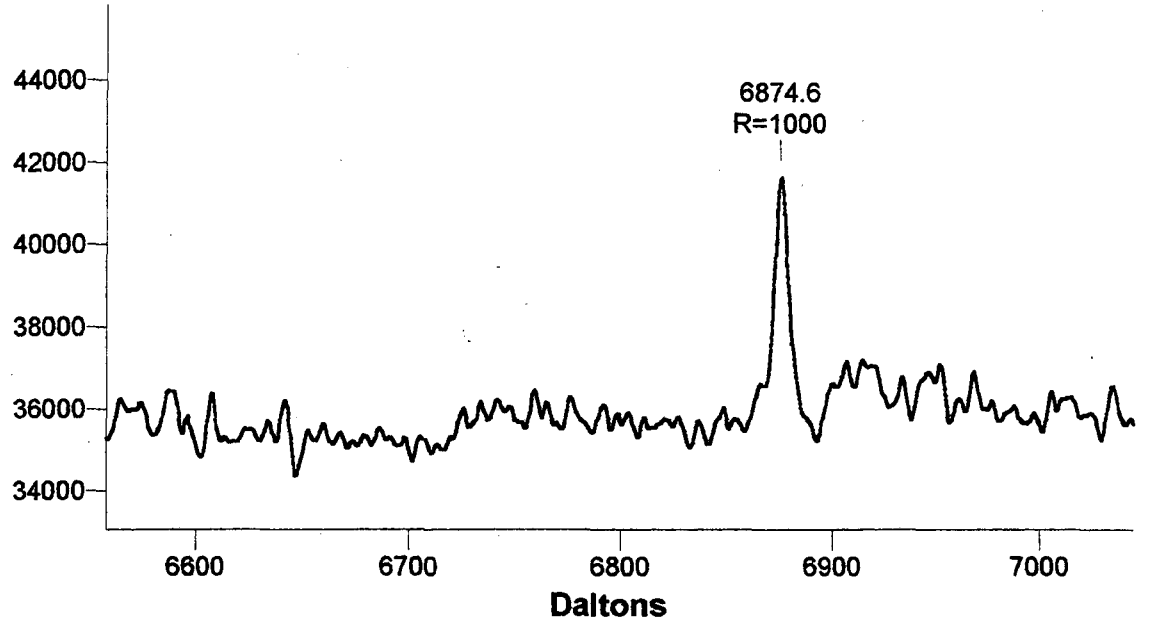

Fig. 1. A 22-base long polynucleotide in 3HPA, Mr = 2000, 386 shots averaged, -25 kV, 400 MHz digitization. The vertical axis is relative ion intensity. (cfgb4.fig)

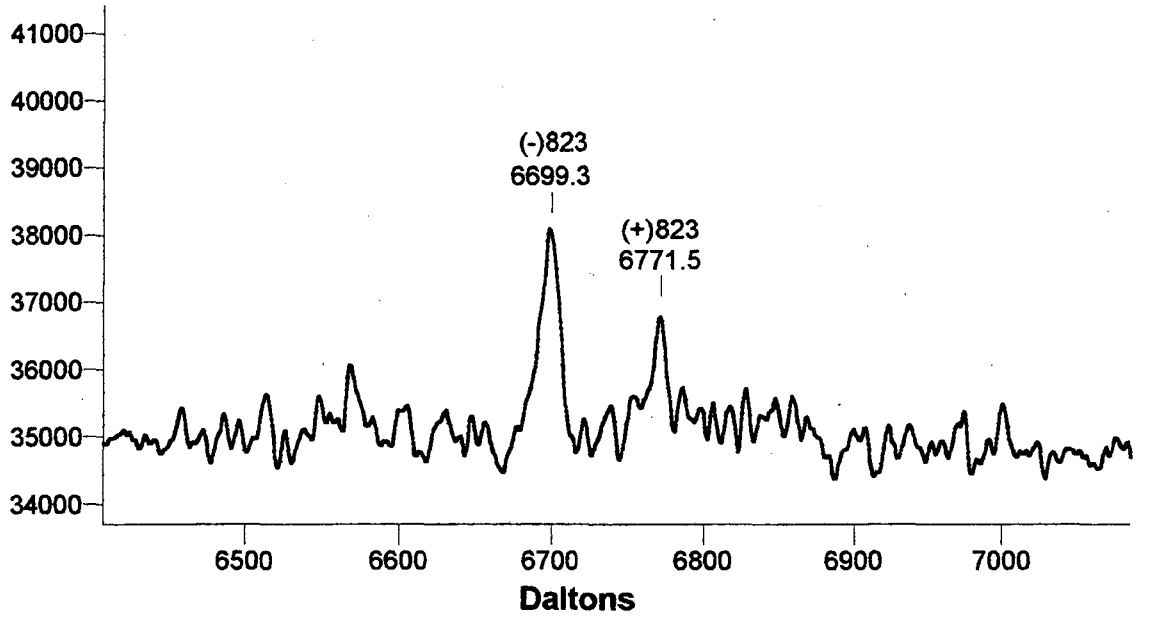

Fig. 2. Separation of the complimentary strands of +/- 823 dsDNA. Matrix = 3HPA, Mr = 5000, 531 shots averaged, 400 Mhz digitization. The calculated mass for -823 is 6701.4 Da and 6768.4 Da for +823. The vertical axis is relative ion intensity. (823fcmpa.fig)

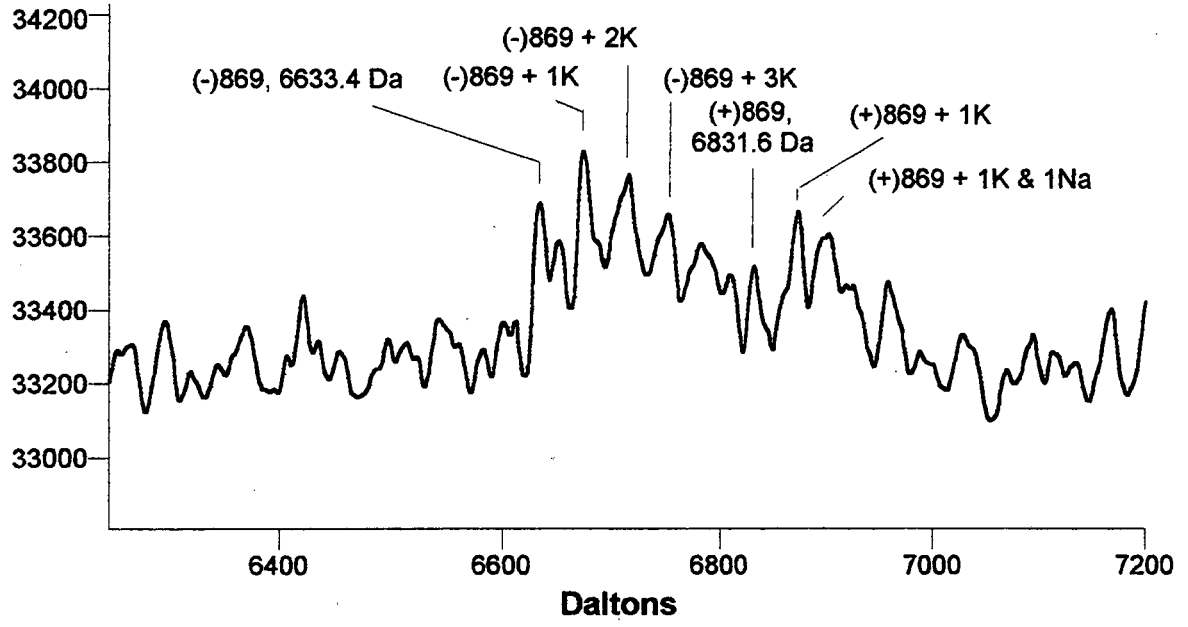

Fig. 3. Separation of the complimentary strands of +/- 869 dsDNA. Matrix = 3HPA, Mr = 5000, 482 shots averaged, -30 kV, 400 MHz digitization. The vertical axis is relative ion intensity. The calculated mass for the -869 strand is 6636.4 Da and 6832.4 Da for +869. (869fcmpd.fig)

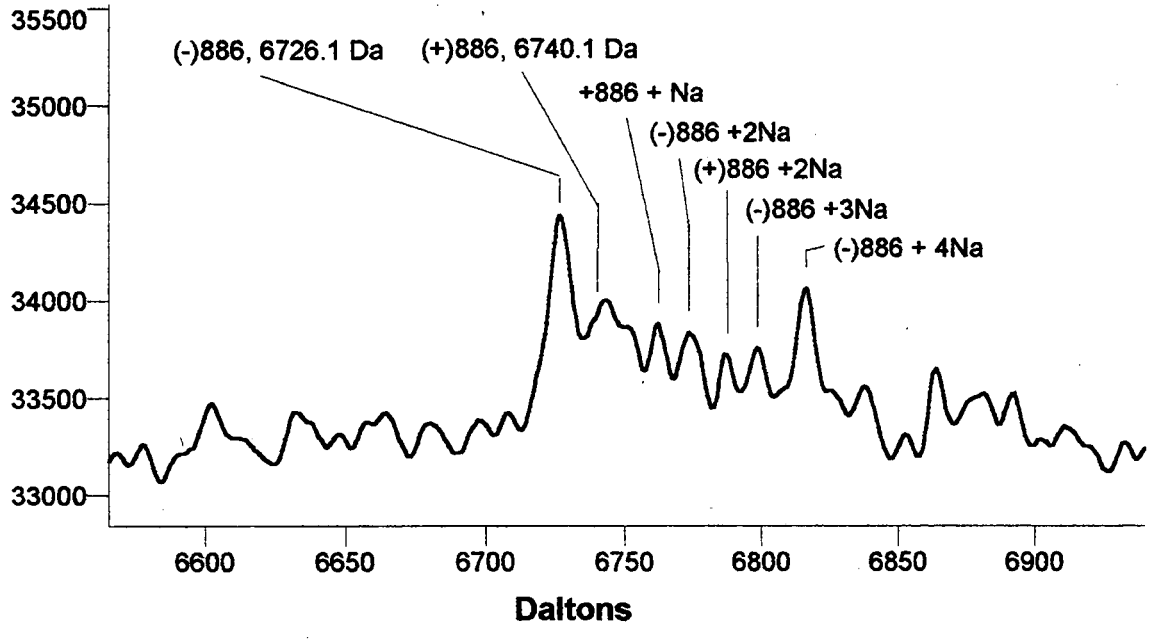

Fig. 4. Separation of the complimentary strands of +/- 886 dsDNA. Matrix = 3HPA, Mr = 5000, 825 shots averaged, -30 kV, 400 MHz digitization. The vertical axis is relative ion intensity. The calculated mass for the -886 strand is 6728.4 Da and 6741.4 Da for +886. (886fcmpd.fig)

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