LASER DESORPTION MASS SPECTROMETRY FOR DNA ANALYSIS AND SEQUENCING

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ABSTRACT

Laser desorption mass spectrometry has been considered as a potential new method for fast DNA sequencing. Our approach is to use matrix-assisted laser desorption to produce parent ions of DNA segments and a time-of-flight mass spectrometer to identify the sizes of DNA segments. Thus, the approach is similar to gel electrophoresis sequencing using Sanger's enzymatic method. However, gel, radioactive tagging, and dye labeling are not required. In addition, the sequencing process can possibly be finished within a few hundred microseconds instead of hours and days. In order to use mass spectrometry for fast DNA sequencing, the following three criteria need to be satisfied. They are (1) detection of large DNA segments, (2) sensitivity reaching the femtomole region, and (3) mass resolution good enough to separate DNA segments of a single nucleotide difference. It has been very difficult to detect large DNA segments by mass spectrometry before due to the fragile chemical properties of DNA and low detection sensitivity of DNA ions. We discovered several new matrices to increase the production of DNA ions. By innovative design of a mass spectrometer, we can increase the ion energy up to 45 KeV to enhance the detection sensitivity. Recently, we succeeded in detecting a DNA segment with 500 nucleotides. The sensitivity was 100 femtomole. Thus, we have fulfilled two key criteria for using mass spectrometry for fast DNA sequencing. The major effort in the near future is to improve the resolution. Different approaches are being pursued. When high resolution of mass spectrometry can be achieved and automation of sample preparation is developed, the sequencing speed to reach 500 megabases per year can be feasible.

2. INTRODUCTION

Since the discovery of the double helix structure of DNA,¹ research on DNA has been a key component of biological and medical research. The major functions of DNA comprise the transmission of information through a genetic code and self-replication. For example, the sequence of three nucleotides in DNA can decide the kind of amino acid to be produced. Due to the complexity of the human genome, and the importance of the DNA function, the Human Genome Project has been initiated and extensively pursued to complete the mapping and sequencing of the whole human genome.² During the past ten years, tremendous progress has been made for genome mapping.³ Successful cloning of very large pieces of DNA in yeast artificial chromosome (YAC) and discovery of sequence-tagged sites (STS) and polymerase chain reactions (PCR) for mapping are just two examples. However, the successes of new sequencing methods to speed up the sequencing rate have been relatively limited. One of the major challenges for the Human Genome Project is to increase the sequencing output by a factor of 100 compared to the present gel sequencing method.⁴

Current gel electrophoresis methods of DNA sequencing, such as the DNA ladder products preparation by Sanger⁵ and Maxam-Gilbert⁶ have proven very useful for sequencing small DNA segments. These methods of sequencing usually involve labeling fragments of DNA for identification following gel electrophoresis.

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Either radioactive labels such as ^{32}P or ^{35}S or chemical labels such as fluorescent dyes are currently used. At the present time, routine DNA sequencing in most laboratories relies on gel electrophoresis to separate DNA fragments labeled with radioactive isotopes. The fragments are generated either chemically or enzymatically to represent all possible positions of each of the four nucleotides (A, G, C, and T). Following electrophoresis, the radioactive labels are located by autoradiography. This method is extremely time consuming. With radioactive labeling, four lanes of the gel are usually required to separate the fragments from a given DNA segment that terminates in A, G, C, or T. Substitution of fluorescent labels for radioisotopes allows the DNA fragments to be detected continuously during electrophoresis. This fluorescent method⁷ usually employs four different labels. Using these four labels, all four types of DNA fragments (A, G, C, and T) can be run in one electrophoresis lane. The uncertainty in comparing the label position in four adjacent lanes of the gel is eliminated. However, this method is limited to spectroscopically resolved dyes which must not lead to differential effects on the electrophoretic mobility of DNA fragments. The possible overlap of fluorescence emissions from different dyes and the modification of the molecular weights of the DNA fragments due to the tagging with dyes having different molecular weights sometimes causes complexity and confusion in the DNA sequencing. Both radioactive methods and fluorescent dye labeling methods for DNA sequencing require the use of the time-consuming gel electrophoresis method. Both have difficulty resolving oligonucleotides of lengths greater than 600 nucleotides and are most effective in the ~ 300 nucleotide range. Recently, capillary gel electrophoresis⁸ and ultra-thin gel electrophoresis⁹ have been pursued to speed up the separation process. Nevertheless, the preparation of the gel and the limitation of the speed for separation by gel makes the search of alternative methods become critically important for fast human genome sequencing.

It is naturally desirable to consider a mass spectrometry approach for DNA sequencing since the development of mass spectrometry for large bio-organic molecules detection has already aroused major interest during the past decade.¹⁰ The most difficult problem in mass spectroscopy of large bio-organic molecules is maintaining the integrity of the molecules. In general, when the molecular weight increases, the fragmentation ratio also increases. Thus, it is extremely difficult (or impossible) to carry out DNA sequencing by a mass spectroscopic method unless the fragmentation of DNA segments can be mostly eliminated.

In order to overcome this difficulty, capillary electrophoresis mass spectrometry¹¹ and resonance ionization mass spectroscopy¹² are being evaluated for DNA sequencing. The capillary electrophoresis mass spectrometer method still relies on slow electrophoresis and the mass spectrometer simply serves as a detector. The approach with resonance ionization is to tag various isotopes of a selected metal element, such as Sn, to DNA fragments with selected base (A, G, C, T) termination. Gel electrophoresis is used to spatially separate the DNA fragments of different sizes. Finally, resonance ionization mass spectroscopy of Sn atoms sputtered from the gel surface by an ion microprobe beam is used to provide isotopically selective detection of the tag element. From this isotopically selective detection of the metal element, which is used as a tag, the sequencing of DNA would be achieved. Since this method is set up for metal element detection, fragmentation is not a concern. On the other hand, the need of gel electrophoresis in this scheme can be a major disadvantage for fast DNA sequencing.

In order to speed up the sequencing by mass spectrometry, it is desirable to do sequencing by mass spectrometry alone, without the need of gel electrophoresis. Thus it is necessary to achieve "soft" desorption of DNA without breakup of fragile DNA compounds and "soft" ionization without producing fragmented ions. However, in 1987 Hillenkamp and his co-workers¹³ discovered that large protein molecular ions can

be produced by laser desorption without much fragmentation if these biomolecules are mixed with small organic compounds that serve as matrices for strong absorption of a laser beam. This process is now called matrix-assisted laser desorption/ionization (MALDI). The typical preparation technique for MALDI is to dissolve biomolecular samples in solution, then prepare another solution that contains small organic compounds such as sinapinic acid. These two solutions are subsequently mixed and a small amount of solution is placed on a metal plate to dry. After the sample crystallizes, the sample plate is placed in the mass spectrometer for analysis. With the MALDI process, matrix materials strongly absorb the laser energy and quickly become vaporized. Large biomolecules are carried along during the fast vaporization process and some of them become ionized.

Since the discovery of MALDI, many research groups have succeeded in measuring various proteins by MALDI. Observation of protein ions with molecular weights greater than 100,000 Daltons without significant breakup have been reported.¹⁴ MALDI has also been applied to DNA segments, but until recently the success has been limited to very small segments probably due to fragile glycosidic bond. Spengler et al.¹⁵ reported the observation of 8 mer single-stranded DNA ions with detection sensitivity of 10 to 100 picomole. Huth-Fehre et al.¹⁶ reported observations of parent ions of polydeoxyribothymidilic acid. Negative-ion spectra of d(T)₅ and d(T)₁₀ have been reported by Karas and Bahr¹⁷ as well as Borsen et al.¹⁸ Parr et al.¹⁹ pointed out that 2,5-dihydroxybenzoic acid is a much more efficient matrix material for polydeoxyribothymidilic acid than for mixed-base DNA segments. Schneider and Chait²⁰ reported a similar conclusion for several matrices and observed polymer ions of poly-T. Curie and Yates²¹ reported success in detecting negative oligomers of 60 bases by using a mixture of matrices. Recently, Wu et al.²² discovered that 3-hydroxypicolinic acid as a good matrix for mixed-base oligonucleotides and succeeded in detecting oligonucleotides of 67 bases. Williams and his co-workers^{23,24} took a slightly different approach and obtained a positive ion DNA spectra by laser ablation and ionization from a frozen aqueous matrix with a laser wavelength tuned to the electronic resonance excitation of sodium or copper atoms. They observed DNA mass spectra with good resolution. Nevertheless, all results only show the capability of using mass spectrometry for small oligonucleotide detection.

In spite of the successes in detecting small DNA compounds by mass spectrometry, there has been a serious concern whether large DNA segments can ever be detected by mass spectrometry. Romano and Levis²⁵ reported the success of laser vaporization of single-stranded DNA molecules up to 1000 bases. However, the detection was based on gel electrophoresis using radioactive tagging instead of direct ion detection by a mass spectrometer. Although their results indicate that large DNA segments can be desorbed, the possibility to ionize DNA segments and efficient detection still have not been answered. Recently, we succeeded in using laser desorption mass spectrometry to detect single-stranded DNA the size of 500 mer and double-stranded DNA with 500 base pairs. This clearly indicates that laser desorption mass spectrometry can be used for large DNA analysis.

In order to achieve fast DNA sequencing by laser desorption mass spectrometry, in our opinion, the following three conditions must be met: (1) the size of the DNA fragment to be detected should be larger than 300 nucleotides, which has been routinely sequenced by gel electrophoresis, (2) the sensitivity must be high enough to detect a few femtomole for each DNA segment, and (3) the resolution of mass spectra must be sufficiently high for determining the distribution of DNA with different sizes.

We give this example of mass spectrometry for DNA sequencing: if a small single-stranded oligonucleotide with the structure of GAA TTC CGC CGA TAC TGA CGG GCT CCA GGA GTC GTC GCC ACC to be sequenced, Sanger's method will produce four different sets of DNA ladder products with selected terminal nucleotides such as A (adenine), T (thymine), C (cytosine), and G (guanine). These four sets of DNA ladder products are to be mixed with a matrix that is suitable for DNA, and these four different samples, they are subsequently placed into the mass spectrometer for detection. Figure 1 is the illustration of the concept for using MALDI-MS for DNA sequencing. The time required for running gel electrophoresis is usually in terms of hours. However, the time for analyzing a DNA sample by mass spectrometry is typically in the range of a few hundred microseconds.

3. RESULTS AND DISCUSSION

The linear time-of-flight mass spectrometer that was designed and constructed at Oak Ridge National Laboratory for most of the DNA work in the past three years is shown in Fig. 2. A Nd:YAG laser capable of delivering four different wavelengths (i.e., 1064 nm, 532 nm, 355 nm, and 266 nm) was used for laser ablation. Typical laser fluence used was less than 200 mJ/cm² to prevent any possible production of dense plasma or fragmentation. An ion deflector was used to deflect electrons and small ions when necessary, since the large number of desorbed electrons and small matrix ions was often large enough to saturate the electron multiplier. A conversion box with an aluminum foil target was used to receive ions and emit secondary electrons. A Johnston electron multiplier was used to detect secondary electrons from the aluminum foil in the conversion box. The conversion box was installed to reduce the probability of saturation of the electron multiplier by scattered laser light and to prevent the contamination of the first dynode of the multiplier by organic or bio-organic samples. Signals from the multiplier went through a preamplifier and subsequently to a fast digital oscilloscope. TOF waveforms from each laser pulse were transferred to a computer for data storage and retrieval. A telescope was installed to observe the laser spot on the sample during the experiment. A He-Ne laser was used to guide the Nd:YAG laser beam to assure the spot of the sample to be desorbed by the laser beam. We also have a second laser system that includes a Nd:YAG laser pumped dye laser with a frequency-doubling device to do post ionization after laser desorption whenever post ionization is desirable. Our facility has a few special features not generally available in commercial TOF instruments. They are: (1) a carefully designed chamber particularly suitable for applying high voltage. The voltage on our sample plate can reach 45KeV. High ion energy is usually of critical importance for large DNA ion detection, since the production of secondary electrons is close to zero when the ion velocity is below 1×10^6 cm/sec.²⁶ (2) an electron multiplier with a large detection area was used instead of a microchannel plate, since an electron multiplier tends to have a higher gain ($10^7 \sim 10^8$) and is less likely to be saturated due to the special geometric structure of the Johnston multiplier; (3) the facility can be easily modified for the length of the drift tube for post acceleration after ion production, and for both positive and negative ion detection; (4) a magnetic field was used to deflect electrons to reduce background from desorbed electrons when the TOFMS is set for negative ion detection; and (5) the aluminum foil can be easily biased with very high voltage without the serious concern of damaging the expensive electron multiplier or microchannel plate detector. Our major results are as follows.

3.1. Detection of large DNA segments by the discovery of new matrices more suitable for DNA detection by MALDI

MALDI has been used very successfully for protein but not for DNA. One major reason for this shortcoming

is that adequate matrices were not used. We discovered picolinic acid to be a better matrix than 3-HPA for MALDI of DNA.²⁷ Mixtures of ss-DNA of 150 mer and 100 mer have been successfully detected by laser desorption mass spectrometry (see Fig. 3).

Recently, we discovered 3-aminopicolinic acid (3-APA) to be another good matrix for MALDI of DNA.²⁸ 3-APA was successfully used to detect ds-DNA segments of 246 base pairs (bp), which were produced by polymerase chain reaction (PCR) for amplification of PLB129 genomic DNA. Experimental results are shown in Fig. 4. Then we discovered that using the mixture of 3-HPA and PA as a matrix,²⁹ a ss-DNA of 500 nucleotides and a ds-DNA with 500 bp can be detected by laser desorption mass spectrometry (see Fig. 5). Yet another records for DNA detection. The DNA segments were produced by using PCR for amplification of a segment of bacteriophage lambda genome. Since most conventional gel electrophoresis methods for DNA sequencing are limited to a few hundred nucleotides, we considered our results clearly indicate that laser desorption mass spectrometry can detect large DNA segments. This should clarify the tremendous concern about the capability of mass spectrometry for large DNA detection. If MALDI samples can be prepared in smaller sizes, the detection sensitivity can be significantly increased. Thus, we believe the detection sensitivity for large DNA segments can possibly reach one femtomole in the future. Thus, we consider that we have met two of three criteria for using mass spectrometry for DNA sequencing. Namely, the requirements for detecting large DNA segments with good sensitivity have been achieved. The remaining critical problem is to improve the resolution of mass spectra of DNA.

3.2. Studies of the fundamental mechanism of laser desorption mass spectrometry of DNA

Discovery of metastable states of negative DNA ions: Since several groups have reported that a reflectron time-of-flight mass spectrometer is less sensitive in detecting negative ions in large DNA segments than a simple linear time-of-flight mass spectrometer,³⁰ we began to wonder about the existence of metastable states of negative DNA ions. A facility shown in Fig. 6 was constructed to test this existence. When the voltages of V_1 , V_2 , and V_3 were at -3kV, +10kV, and -5kV respectively, neither positive nor negative ions should reach the detector. However, we still observed signals corresponding to DNA analyte in the samples. Thus, we came to the conclusion that negative ions decay into a smaller negative ion and a neutral molecule that can still be detected with the experimental configuration in Fig. 6.³¹ Lifetimes of negative DNA ions and quenching cross sections by the residual gases were also measured (see Fig. 7). These results can have very critical implication. If large negative DNA ions only have a lifetime of less than a few hundred microseconds, then conventional reflectron time-of-flight mass spectrometer (RTOFMS), Fourier Transform mass spectrometer (FTMS), and ion-trap mass spectrometer (ITMS) may not be an adequate instrument for detection of large DNA segments. The limited lifetime of negative DNA ions must be taken into account with any design for DNA analysis or sequencing by mass spectrometry.

Measurements of velocity distribution of desorbed DNA ions: The velocity distribution of desorbed protein has been measured to be about the same as the distribution of the matrix. If a protein has a molecular weight of 100,000 Daltons and the molecular weight of the matrix is 100 Daltons, then the average kinetic energy of protein can possibly reach 1 KeV. If the voltage of the target is at 30 KeV, it will be extremely difficult to get a mass spectrum with resolution better than 100 ($M/\Delta M$). Thus, the broad velocity distribution and energetic biopolymer ions in the MALDI process is one of the major reasons for poor resolution. We measured the velocity distribution of desorbed DNA ions (see Fig. 8). Although the results are somewhat similar to the results of protein, they clearly indicate that the average velocity of larger DNA segments is

lower than the matrix compound.

3.3. Successful detection of restriction enzyme-digested DNA

Laser desorption mass spectrometry has been used to detect the fragments of DNA that were produced by restriction enzyme digestion.³² DNA segments amplified by PCR without further purification can also be detected by MALDI. However, resolution and reproducibility are also worse than for pure DNA. The success of detecting DNA segments from restriction enzyme digestion can possibly lead to fast DNA fingerprinting in the future.

4. CONCLUSION

The mechanism of MALDI of DNA definitely needs more careful study to improve its performance. In order to achieve fast DNA sequencing by laser desorption mass spectrometry, major efforts need to be focused on improving resolution. However, a major breakthrough in resolution can come from either innovative instrumentation design or much better matrices fitted for DNA ionization and desorption. We believe this major breakthrough will come within the next few years.

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Fig. 1 Conceptual mass spectra for DNA sequencing. If we need to sequence the part of the DNA with the sequencing structure of GAA TTC CGC CGA TAC TGA CGG GCT CCA GGA GTC CTC GCC ACC, a primer is needed for producing Sanger's ladder products. With ddNTP, different sizes of DNA with selected terminations are produced. There are five different sizes of DNA with the last base of T. Their sizes are 25 mer, 29 mer, 31 mer, 40 mer, and 41 mer. The number of different sizes of DNA with the last base A, G, and C will be 4, 5, and 4 respectively. If no fragmented ions are produced, the mass spectra can easily be used to construct the sequencing information.

Fig. 2 Schematic of a linear TOF facility in the Photophysics Group at ORNL. Sample substrate can be biased with voltage up to 45kV.

Fig. 3 Negative ion mass spectrum of synthetic 100 mer and 150 mer. Amount of sample loaded was 30 pmol for each oligonucleotide. 2.4 μ mol picolinic acid was used as matrix. Laser wavelength was 266 nm and laser fluence was 170 mJ/cm².

Fig. 4 Negative ion spectrum of a 246 bp DNA amplified from pLB129. Total amount of DNA loaded was about 2 pmol. 1.6 μ mol picolinic acid + 0.2 μ mol 3-HPA was used as matrix. Laser wavelength was 266 nm and laser fluence was 100 mJ/cm². The sequence of one strand of the DNA is 5'-AGGAATTCCA TGGCCTGGAC CCCTCTCTGC CTCTCCTTCC TCACCCTCTA CAAAGGTGAT GCCCCAACC CTGCCTTAGG CTCAGCCCTT ACAGGATCCT GAGCTGGTCC TGCCCTGAAC CCTGAGCTCA GCCTAGGCAT AGCCTCAGGG TGACACCACT GGAAATGTGT TTGTATCTT CAAGCCCCCT CTCCTTCCT CTCCTGCAGG CTCTGTGGCC TCCTATGAGC TGACTCAGCC ACCCTC-3'.

Fig. 5 Negative ion spectrum of a 500 bp DNA amplified from bacteriophage lambda genome. Total amount of DNA loaded was about 2.5 pmol. 2.4 μmol picolinic acid + 0.3 μmol 3-HPA was used as matrix. Laser wavelength was 266 nm and laser fluence was 200 mJ/cm^2 .

Fig. 6 Modified MALDI-TOF instrument with the ability to check the existence of metastable negative DNA ions. The ability to independently vary the sample, flight tube, and detector voltage allows the neutral and ionic species to be separated.

Fig. 7 Pressure dependence data for the 4- and 7-mer in 2,5-dihydroxybenzoic acid. The slope of the fitted line determines the collisional cross section, and the intercept determines the zero-pressure lifetime.

Fig. 8 Velocity distributions of desorbed negative ions of $d(T)_{20}$, $d(T)_{40}$, $d(T)_{60}$ and 2,5-dihydroxybenzoic acid.