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Approaches to functional genomics: potential of matrix-assisted laser desorption ionization–time of flight mass spectrometry combined with separation methods for the analysis of DNA in biological samples

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Abstract

MALDI–TOF MS has potential as a valuable technique in DNA mapping studies and may well be complementary to other approaches to DNA analysis such as gel electrophoresis and sequencing. This study used 2,6-dihydroxyacetophenone (DHAP) mixed with diammonium hydrogen citrate (DAHC) as the matrix. In addition, recent technical advances such as time lag focussing (TLF) and better selection of matrices (such as 3-hydroxypicolinic acid (3 HPA) and picolinic acid (PA)) extended the range of DNA fragments that can be studied by this approach. The following samples were investigated: Poly-T mixture (dT 15, 19, 20, 25, 74 and 75), plasmid pBR322 derived oligonucleotides (10, 11, 12, 13, 14, 15, 19, 20 and 50 nucleotides long) and DNA fragments of 25, 36 and 37 base pairs corresponding to a fragment in the restriction map for the gene corresponding to the hexon protein of Adenovirus 2 and 5. The results were contrasted with similar analyses performed by ion-paired reversed-phase HPLC coupled to on-line electrospray mass spectrometry. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Functional genomics represents a wide-ranging opportunity for the analytical chemist and is directed at the development of global experimental approaches to assess gene function by making use of reagents provided by structural genomics [1]. With this goal in mind we are exploring methods for rapid chemical mapping of large numbers of DNA samples. The chemical mapping approach can be directly analogous to peptide mapping of proteins in which the macromolecule (DNA, protein) is fragmented by either chemical or enzymatic means and the resulting mixture of fragments are separated electrophoretically or chromatographically. In the analysis, a shift in mobility can be correlated with a difference in size or chemical structure. Such approaches will be particularly useful in post-genome research where large numbers of closely related DNA sequences need to be examined. An example of such studies includes the examination of genetic stability of an

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organism during a fermentation process and the exploration of mutational events during cellular transformation. While not as specific as DNA sequencing approaches, the use of DNA mapping has the advantage that the analysis can be concentrated on the small segment of DNA that contains the chemical difference. In this context MALDI–TOF MS would appear to be particularly suitable in that it is a rapid mass spectrometry procedure that can be used for mixtures of heterogeneous biopolymers. Also, MALDI–TOF MS has been recently combined with both LC and electrophoretic separations in an efficient off-line procedure [2]. Recent publications have also shown that MALDI–TOF MS can be applied to oligonucleotide samples, either prepared by chemical synthesis or enzymatic reactions [3–7].

Our laboratory has recently explored the use of LC–electrospray mass spectrometry (MS) in the analysis of DNA samples [2]. These studies demonstrated that the combination of a reversed-phase separation with on-line mass spectrometry is powerful in elucidating chemical differences in shorter oligonucleotides such as synthetic probes, PCR primers or antisense therapeutics (optimally 10–20 nucleotides). With this publication, we will explore the use of MALDI–TOF MS in combination with separations to analyze longer and double stranded DNA fragments that are more typical of biological samples. This methodology also has potential for shorter oligonucleotides in that MALDI–TOF MS often provides complementary data to that of electrospray mass spectrometry.

2. Experimental

2.1. HPLC

The HPLC separation was performed on a Hewlett-Packard 1090 Liquid Chromatography system equipped with DR5 ternary solvent delivery system, diode array UV/VIS detector (DAD), autosampler and heated column compartment (Hewlett-Packard, Wilmington, DE). All HPLC separations were done using a YMC, (Wilmington, NC) 3 μm particle, 120 Å pore size ODS-AQ C_{18} reversed-phase column 250 mm×2.1 mm I.D. Flow rates were at 0.2 ml/min. Injection volumes were 1–2 μl. The column temperature was maintained at 35°C throughout the separation. UV absorbance was monitored at 269 nm with 10 nm slit width and reference at 480 nm with an 80 nm slit width. As described below in the Discussion, two main gradient solvent systems were used: 100 m*M* triethylammonium acetate (TEAA) pH 7.0/acetonitrile and 400 m*M* hexafluorisopropanol (HFIP)/methanol. The TEAA mobile phase was prepared with a dilution of a 1 *M* preformulated commercial buffer (Fluka BioChemika, Buchs, Switzerland) to 100 m*M*. The pH was measured to ensure pH 7.0. For this solvent system, the ''B'' buffer consisted of 50% acetonitrile, 100 m*M* TEAA. The HFIP mobile phase was prepared as a stock solution of 800 m*M* adjusted to pH 7.0 with triethylamine. Approximately 1.2 ml of TEA is needed to titrate 1 l of 800 m*M* HFIP to pH 7. This stock solution was diluted to 400 m*M* with water for the ''A'' solvent and with methanol for the ''B'' solvent. The solvents were degassed ultrasonically.

2.2. Electrospray ionization MS

Electrospray ionization mass spectrometry was done on a Hewlett-Packard 5989B Quadrupole Mass Spectrometer equipped with extended mass range, high energy dynode detector (HED) and a Hewlett-Packard 59987A API-Electrospray source with high flow nebulizer option. Both the HPLC and MS were controlled by the HP Chemstation software allowing simultaneous instrument control, data acquisition and data analysis. The high flow nebulizer was operated in a standard manner with N_2 as nebulizing (1.5 l/min) and drying (15 l/min at 300°C) gases. The system was operated in negative ion electrospray mode. The use of the high flow nebulizer negates the need for flow splitting or for scavenger gases such as oxygen or SF_6. Typical source high voltage settings were V_{cap}=4000 V, V_{cyl}=3500 V and V_{end}=4000 V. Typical source internal lens voltages were CapEx=−191 V, ESSkim1=−30 V, Ion Guide V_b=−5 V, Ion Guide V_e=46 V, and Entrance Lens=−75 V. The HED detector was utilized with 10 kV bias voltage and electron multiplier voltage of 2500 V. MS Data was acquired in raw scan mode scanning from 500 to 2000 amu at an acquisition rate of 1.0 Hz at 0.15 amu stepsize. Data was filtered in the mass domain with a 0.05 amu mass filter and in the time domain

with a 0.05 min gaussian time filter. Unit mass resolution was maintained for all experiments, allowing unambiguous identification of −1 and −2 charge states.

2.3. MALDI–TOF MS

Matrix-assisted laser desorption time-of-flight (MALDI–TOF MS) mass spectrometry was performed on a HP G2030A system. All spectra shown were obtained in the negative ion-mode. The matrix used was 2,6-dihydroxyacetophenone (DHAP) in ethanol mixed with diammonium hydrogen citrate (DAHC). One part DNA (total 20 pmol) was mixed 1:1 with 4 parts matrix (DHAP/DAHC) to form crystals after vacuum drying. A sample of 1 μl was placed on the mesa on the probe tip, vacuum dried and subsequently put into the TOF mass spectrometer for analysis. In general, mass spectra from 50 to 100 laser shots are summed to produce the final spectrum.

2.4. Time-lag-focusing (TLF) MALDI–TOF MS

The time-lag focusing MALDI–time-of-flight mass spectrometer has been described elsewhere [7]. Briefly, it features a four-plate source design, pulsed ion extraction for time-lag focusing, and a one-meter linear flight tube. The ions are generated by using a 3 ns pulse width laser beam from a nitrogen laser at 337 nm (Laser Science Inc., VSL 337 ND, Newton, MA). A dual microchannel plate detector is used for ion detection and a Hewlett-Packard MALDI data system combined with a LeCroy 9350 digital oscilloscope with a sampling speed of 1 Gigasample/s is used for mass spectral recording and data processing. All spectra shown were obtained in the positive ion-mode. In general, mass spectra from 50 to 100 laser shots are summed to produce the final spectrum.

3-Hydroxypicolinic acid (3 HPA) [8] and picolinic acid (PA) were used as matrices for MALDI analysis of the DNA samples. The 3 HPA matrix was saturated in 33% CH_3CN/H_2O with the aid of vortex mixing, centrifuged, and part of the supernatant solution was transferred to another vial containing regenerated H^+ activated ion-exchange beads (Dowex 50 W-X8, 20–50 mesh). This H^+ ion-exchanged 3 HPA was used to form the first matrix layer. The second matrix layer was prepared by using a mixture of 0.5 *M* PA in 33% CH_3CN/H_2O and supernatant 3 HPA solution in 1:4 (v/v) ratio. DNA was prepared by dissolution in pure water with the addition of NH_4^+ activated ion-exchange beads.

To prepare the sample on the probe tip [9], 1 μl of 3 HPA matrix solution was first loaded on the probe tip and allowed to dry, the crystals were crushed to form the first matrix layer, and then 1 μl of the second layer solution was applied on the top of the first layer. To prepare the second layer solution, 1 μl of ion-exchanged DNA solution, 1 μl of 0.5 *M* diammonium hydrogen citrate and 3 μl of the second layer matrix solution were mixed. As the second layer was drying the solution was stirred on the probe tip using a micro pipette tip until small crystals formed.

2.5. Synthetic oligonucleotide preparation

Homopolymers of thymidine (poly-T) and oligonucleotide sequences derived from the plasmid pBR322 were synthesized by conventional synthesis chemistry using Cruachem (Dulles, VA) chemicals and instrument. Adenoviral sequences were obtained from Operon Technologies (Alameda, CA). After cleavage and deprotection the oligonucleotides were purified by HPLC, desalted with Nap 10 Sephadex G-25 spin columns (Pharmacia, Piscataway, NJ) and lyophylized. The oligonucleotides were resuspended in deionized water to 500 pmol/μl stock solutions and further diluted to 20 pmol/μl for each experiment.

3. Results and discussion

3.1. Electrospray versus MALDI–TOF MS analysis of synthetic oligonucleotide samples

As the first part of our exploration of the use of MALDI–TOF MS for DNA analysis, we analyzed shorter oligonucleotides produced by chemical synthesis. The use of such samples allowed us to optimize the analytical system as well as carry out a comparison with the previous LC/electrospray MS studies to explore the differences in both electro-

spray/quadrupole versus MALDI–TOF MS and the differences between on-line versus off-line MS detection [2]. A technological hurdle for the application of MS to analysis of DNA samples lies in the relatively low molecular weight range available to gas phase analysis of these polyanionic samples. Although, recent studies [10,11] have extended the range to ~500 nucleotides, if one requires useful mass data then the operating range is, however, limited to less than 100 nucleotides in most experimental situations. In contrast biological studies typically involve DNA sequences of much higher molecular weight. For example, PCR reactions typically produce fragments of several hundred nucleotides in length and differences in structure are studied by DNA sequencing. An exception would be procedures such as mini-sequencing (oligo specific priming) in which a DNA mutation is detected by the use of specific primers that allow the production of a short sequence; such sequences can then be readily studied by MALDI–TOF MS. This procedure requires specific knowledge of the mutations prior to the analysis, whereas the mapping procedures can be designed to enable the detection of unknown nucleotide substitutions.

We are exploring a strategy for comparative DNA mapping in which LC/electrospray MS will be used for the analysis of shorter oligomers and MALDI–TOF MS for fragments up to 150 nucleotides. In this approach, longer oligonucleotides will be examined by MALDI–TOF MS and then cleaved to fragments which are amenable to high efficiency separations and subsequent high mass accuracy analysis that will be required for structure determination. The appropriate selection of restriction endonucleases to generate the shorter oligonucleotides can be predicted by commercially available software (e.g., DNASIS, Hitachi Software Engineering Co, Ltd.). We believe that separations will play a key role in the DNA mapping approach in steps such as isolation of a DNA fragment containing an altered nucleotide sequence, desalting the sample for MS analysis and for reducing the sample heterogeneity to the level that suppressed ionization of sample components is minimized.

Fig. 1 shows a typical separation of oligonucleotides (polythymidine or poly-T) by reversed-phase HPLC and the use of on-line electrospray MS. Poly-T was chosen as a chemically stable, model oligonucleotide system, in which the effect of molecular weight differences on the separation process could be explored in the absence of sequence variation. This figure shows that the LC method can readily separate oligonucleotides up to 25 nucleotides in length. Using wide pore silica based column packing materials we have shown resolution of double stranded DNA fragments up to 1300 bp in length (manuscript in preparation). With the power of on-line MS, the mass of each peak in Fig. 1 can be calculated by deconvolution of the envelope of ions caused by salt adduction to the polyphosphate backbone of the oligonucleotide. Mass analysis resulting from ESI–quadrupole MS analysis is currently limited to oligonucleotides of less than approximately 30 nucleotides [2].

Fig. 2 shows the corresponding analysis of the poly-T mixture by MALDI–TOF MS without separation by reversed-phase HPLC. Unlike electrospray MS, Fig. 2 shows that MALDI–TOF MS can give a mass measurement for large oligonucleotides, although the mass resolution was insufficient to give baseline resolution of the signals for the 74 and 75 nucleotide samples. The relatively broader peaks could be caused by salt adduction as well as some decomposition of the sample during ion acceleration in the MALDI source [12]. A small amount of the doubly charged ions are also observed, but unlike electrospray MS, an envelope of charged ions is avoided with the result that good sensitivity (4 pmol on the probe tip) is observed for a sample of mass range of ~22,000 Daltons. In Fig. 2 the MALDI–TOF MS analysis of the poly-T mixture (range: 15–75 nucleotides) shows that all components can be measured in a single run without the need of a fractionation step. If poly T15 and T25 are used as internal standards, the following mass measurements for the $(M-H)$ negative ions were obtained (theoretical values in parentheses): T15, 4500.0 (4500.0); T19, 5716.7 (5716.7); T20, 6019.2 (6020.9); T25, 7541.9 (7541.9); T74, 22476.0 (22447.5); T75, 22764.7 (22751.7). Although MALDI–TOF MS is not usually viewed as a quantitative technique, a visual comparison of Figs. 1 and 2 indicate that both MS approaches are giving similar sensitivities to the different sample components. Since decomposition of the oligonucleotide sample is a concern in MALDI–TOF MS, it is useful to have a comparison with the electrospray MS data as any fragmentation

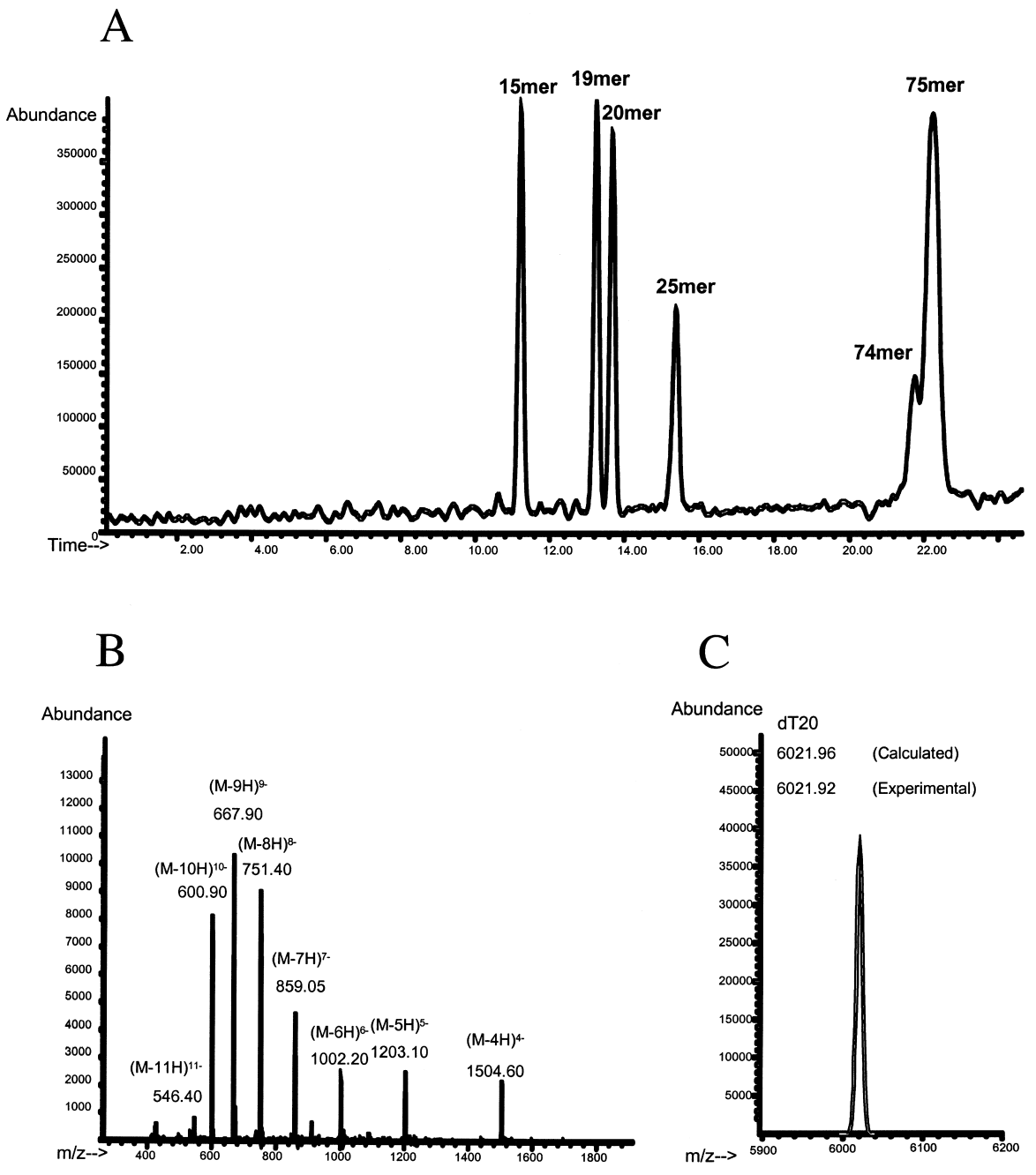

Fig. 1. LC–ESI/MS applications using Poly-T mixture. Conditions are described in the Experimental Section.

would probably not be observed to an equal extent in both ionization processes. An inspection of Figs. 1 and 2, for example, shows that while some fragmentation probably occurs, it is at an insignificant level in this MALDI–TOF MS analysis.

3.2. The use of MALDI–TOF MS to analyze DNA sequences of biological relevance

Plasmid mapping is an important quality control procedure in the biotechnology industry as it is used

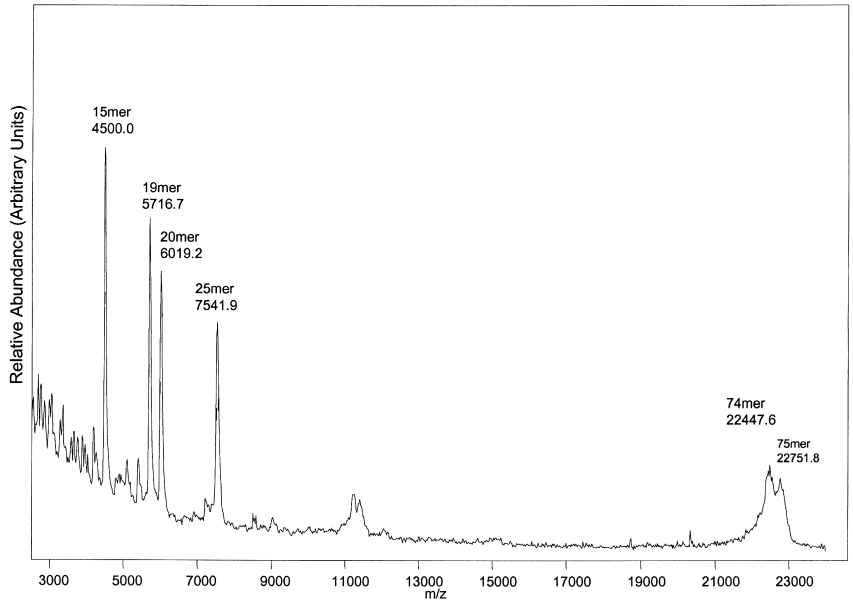

Fig. 2. MALDI–TOF MS applications using Poly-T mixture of short (T 15, 19, 20, 25) and longer (T 74, 75) oligonucleotides. Conditions as in the Experimental section with DHAP/DAHC as the matrix.

to check on the integrity of the gene construct used in the recombinant-DNA manufacturing process. In this analysis the plasmid is digested with a restriction enzyme and the fragments are separated by gel electrophoresis and compared with a reference material. A weakness of this approach is that typically only gross insertion/deletion events are detected as single nucleotide mutations often do not lead to observable differences in electrophoretic mobility, unless the substitution alters one of the enzyme cleavage sites. By contrast our proposed DNA mapping procedures could detect a base substitution as the mass information resulting from MALDI–TOF MS analysis could be used to detect any changes in composition. As a trial experiment as shown in Fig. 3, we performed a MALDI–TOF MS mass measurement on a mixture of 8 fragments (4 pmol each) derived from the sequence of the plasmid pBR322. With the 10 and the 15 nucleotide fragments as internal standards for Fig. 3A the following negative ion ($(M-H)^-$) masses were obtained (theoretical values in parentheses): 10, 3083.0 (3083.0); 11, 3383.4 (3387.2); 12, 3713.1 (3716.4); 13, 4016.6 (4020.6); 14, 4308.5 (4309.8); 15, 4623.0 (4623.0); 19, 5837.6 (5834.8); 20, 6125.6 (6124.0).

A demonstration of the potential of MALDI–TOF MS to detect single base mutations is to compare this mass data with the theoretical differences in mass of the four common nucleotides (A, 313.2; T, 304.2; C, 289.2; G, 329.2). With a typical internal standard mass accuracy of better than 0.02%, as was achieved in the experiment described above, it should be possible to detect the minimum mass difference between a pair of nucleotides (9.0 amu) if the weight of the oligonucleotide is less than 45,000 amu or approximately a 120 mer. Thus, this demonstrates that MALDI–TOF MS has potential in the analysis of plasmid maps, although future work is necessary to improve the sensitivity, mass resolution and establish the limit of the technique for complex mixtures of oligonucleotides.

3.3. MALDI–TOF MS developments

Recently, time lag focusing (TLF) has been introduced as a improvement to MALDI–TOF MS which has allowed a significant improvement in the degree of resolution in mass analysis of biopolymers, such as proteins and oligonucleotides [13]. In preliminary experiments, we have also found that TLF

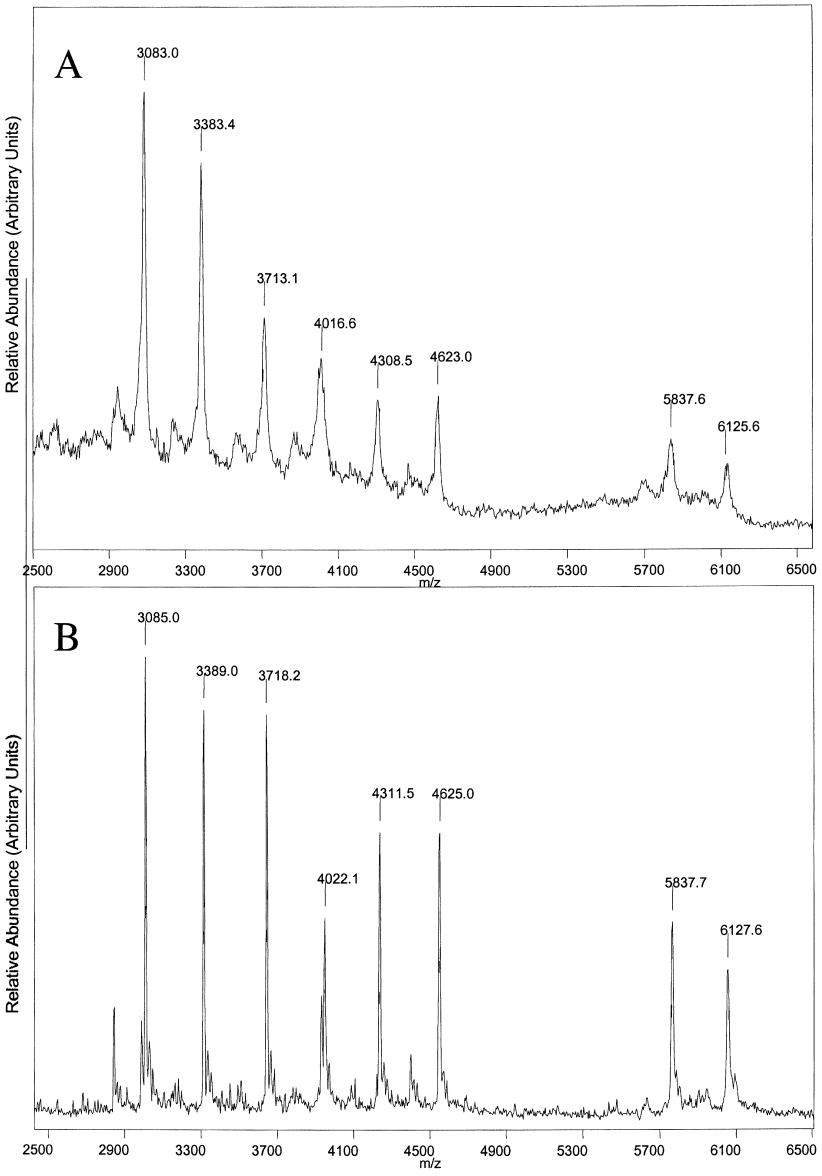

Fig. 3. MALDI–TOF MS applications using a mixture of plasmid pBR322 derived oligonucleotides (10, 11, 12, 13, 14, 15, 19, 20 nucleotides) without time-lag-focussing (A, − ions) and with time-lag-focussing (B, + ions). Conditions as in Fig. 2.

can result in a significant improvement in the mass analysis of our DNA samples, for example, Fig. 3B shows the improved result for analysis of the positive ion ($(M+H)^+$) plasmid pBR322 fragments relative to part A of the figure. Fig. 4 shows the analysis of a 50 base oligonucleotide derived from the same sequence at a level of 50 pmol (part A) and 8 pmol (part B). The improved resolution of 1535, shown in Fig. 4A, can be compared with a typical resolution of 150–200 for this oligonucleotide in a corresponding MALDI–TOF MS system without TLF (data not shown). We are currently exploring the effect of this improvement in mass resolution and mass accuracy to perform DNA mapping studies on different sam-

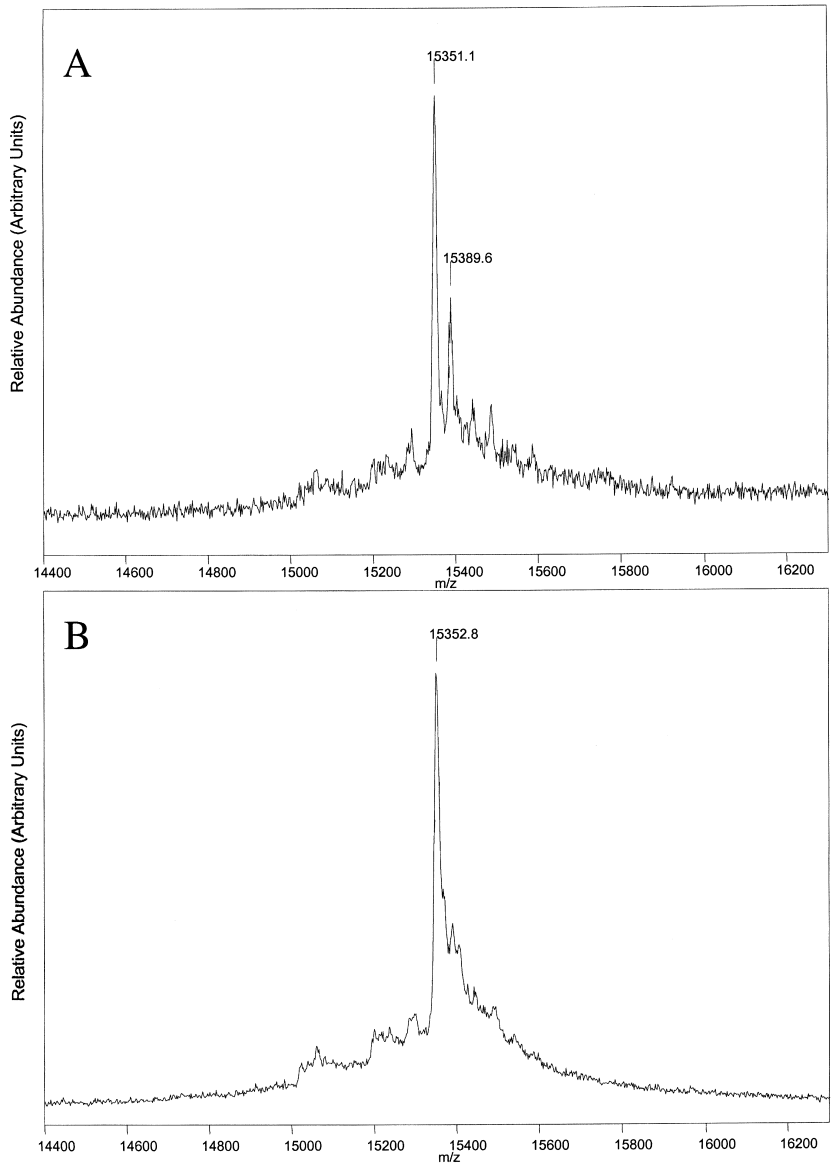

Fig. 4. Time-lag-focusing mass spectrometry applications using a plasmid pBR322 derived oligonucleotide 50 nucleotides long at 50 pmol (A) and 8 pmol (B). See Experimental section for description of details.

ples. In general a low level sample shows a significantly broader mass peak (as in Fig. 4B), so it can be seen that salt adduction is still a problem for DNA analysis, particularly if the sample is derived from a biological extract. We are currently working on procedures such as microdialysis and ion-exchange of the sample with volatile buffers.

3.4. Analysis of adenoviral gene therapy vectors

In a recent publication [14] we described the development of reversed-phase HPLC assays for the protein components present in adenovirus Type 5 samples that have been modified for gene therapy studies. One example is the use of growth factors

delivered via adenoviral vectors to treat coronary disease; the expectation being that the engineered viral DNA will express angiogenic factors that may increase blood flow by stimulating the growth of new blood vessels in the heart. In these studies a variant adenovirus, Type 2 is used as a control relative to the closely related Type 5 virus that is used in the gene therapy. In a quality control environment it is important to analyze cells infected with the gene therapy virus for presence of the growth factor gene and absence of viral genes required for replication. The degree of certainty of an analytical program is significantly increased if the protein assays described above are supplemented by appropriate DNA assays.

One such potential test examines the presence of DNA coding for the hexon protein, which is the

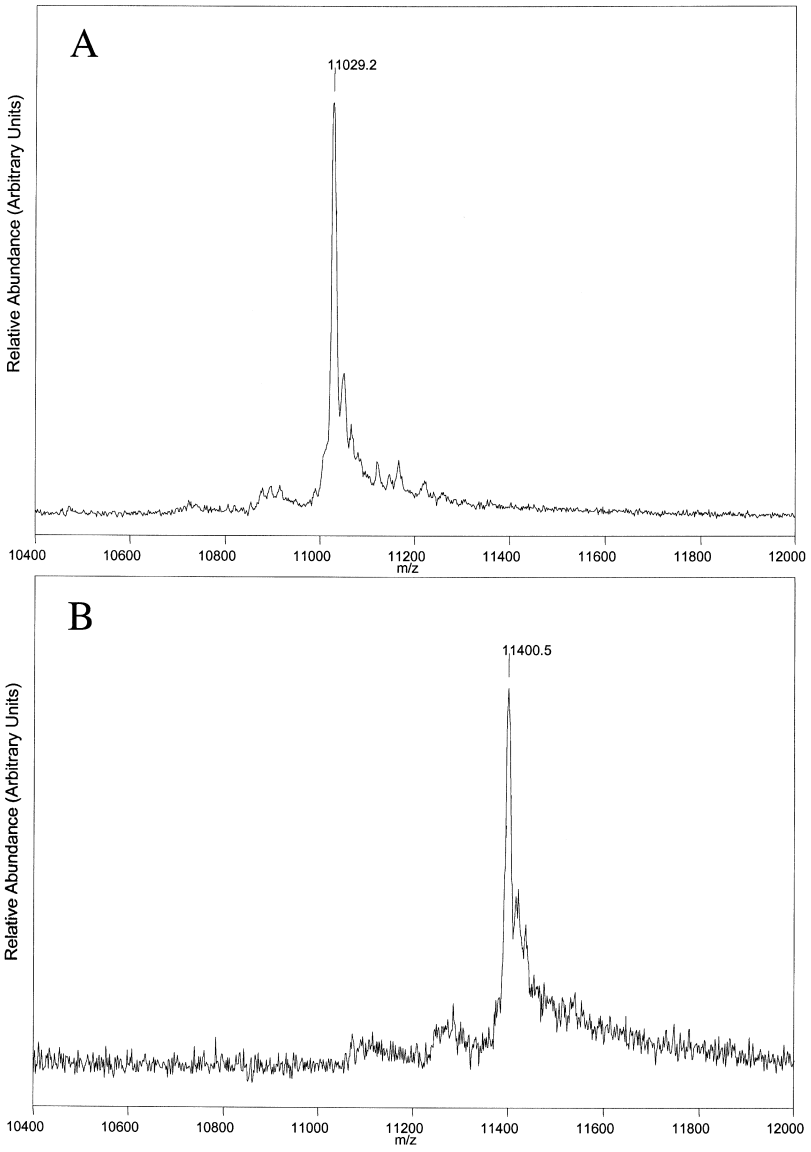

Fig. 5. MALDI–TOF MS analysis of DNA fragments of 36 residues (5′- TGA-TGC-CGC-AGT-GGT-CTT-ACA-TGC-ACA-TGC-CGG-GCC-3′) and 37 residues (5′- TGG-CCC-GAG-AGT-TGC-ATG-TAA-GAC-CAC-TGC-GGC-ATC-A 3′) corresponding to a fragment in the restriction map for the gene corresponding to the hexon protein of Adenovirus 5.

major coat protein of the virus and contains structural differences between Adv 2 and 5 and thus is a good probe for QC of the manufacturing process. Fig. 5 shows the TLF MALDI–TOF result for analysis of a key nucleotide fragment that would be generated in a restriction map (BstNI) of the gene coding for the hexon structural protein. Since the digest is performed on double stranded DNA, two species would be expected in the MS analysis due to the dissociative effects of the laser. A feature of some restriction enzymes, such as BstNI, is that an 'overhang' is produced, in this case the double stranded product has one of the complimentary DNA fragments longer by a single nucleotide (n vs. (n+1)-

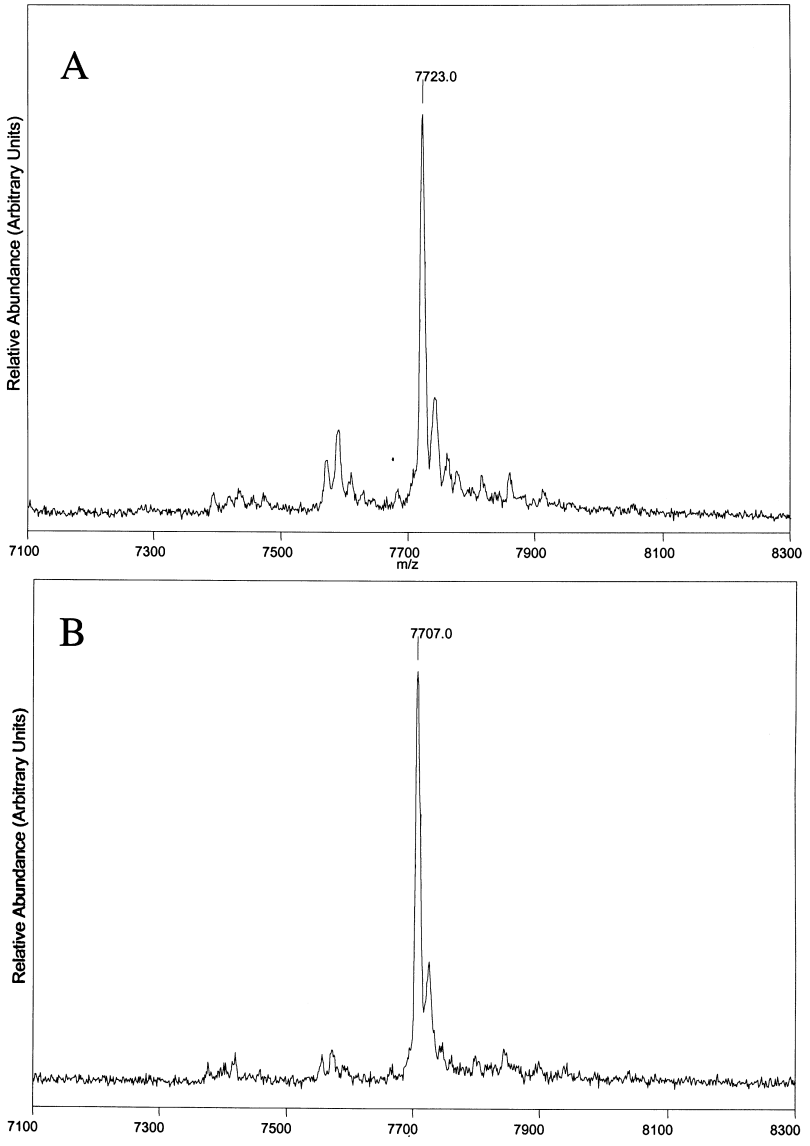

Fig. 6. MALDI–TOF MS application using 25 base DNA fragments in part A (5′- GCG CGG TTC ACC CT**G** GCT GTG GGT G -3′) and part B (5′- GCG CGG TTC ACC CT**A** GCT GTG GGT G -3′) corresponding to the DNA sequence coding for a region of the hexon protein of Adenovirus 2 and 5, respectively.

mer, in this case 36 vs. 37 residues). This difference can be exploited in the analysis in that the mass measurements of the two fragments are further differentiated by the mass difference of the 'overhang' as is shown in Fig. 5 with a mass of 11029 and 11400 (36 vs. 37 mer).

Fig. 6 shows the TLF MALDI–TOF analysis of a 25 base pair fragment which contains one of the sequence differences between the hexon genes of the two adenoviral strains (a replacement of a G residue with A). In this case the analysis is simplified by showing the analysis of the two oligonucleotides resulting from a single base substitution in only one of the strands. In addition, the complimentary strand could also be used to detect the base change. This result shows that the resolution of the instrument is sufficient to distinguish two DNA fragments of the same length but with a change in composition (7723 vs. 7707, difference of 16 for both experimental and theory). Such an analysis is generally not possible for the gel electrophoresis approach.

4. Conclusions

The study described in this publication has demonstrated that MALDI–TOF MS has potential as a valuable technique in DNA mapping studies. Other authors have studied the use of MALDI–TOF MS for DNA sequencing [13], PCR products [15], and thus the studies described here are complimentary. In addition, recent technical advances such as TLF and better selection of matrices will allow us to extend the range of DNA fragments that can be studied by this approach. Future studies will be directed at better methods to cleave DNA into fragments that have a size appropriate for MALDI–TOF MS mass measurement.

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