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Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: a powerful tool for the mass and sequence analysis of natural and modified oligonucleotides

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ABSTRACT

We report the analysis and characterization of natural and modified oligonucleotides by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The present technology was highly improved for this class of compounds by using a new matrix, 2,4,6-trihydroxy acetophenone, together with di- and triammonium salts of organic or inorganic acids to suppress peak broadening due to multiple ion adducts. This methodology can be used in combination with time dependent degradation of oligonucleotides by exonucleases as powerful tool to determine sequence compositions.

INTRODUCTION

Today synthetic chemistry and molecular biology allow the construction of large and complex molecules for which simple methods that enable the analysis and unambiguous identification of these compounds are often not available. Recently MALDI-TOF MS was demonstrated by Karas and Hillenkamp (1,2) to be an excellent tool for the mass determination of high molecular weight biomolecules such as proteins (< 500000 Dalton). The principle of this technique is based on laser pulse induced (260–337 nm or longer wavelength) desorption and ionization of the molecules which are embedded in a large excess of a crystalline matrix. This process is very mild and leads to the desorption and ionization of mainly intact molecules from which usually no fragmentation products are observed. The desorbed, charged molecules are accelerated in a constant electrical field and are detected at the end of a linear tube (Figure 1). The time difference between desorption by a short laser pulse and detection is proportional to the square root of the mass/charge ratio. In addition to successful analysis of peptides and proteins, this technique proved to be useful for other classes of compounds such as oligosaccharides, various oligo- and polymers, lipids, and dyes with a sensitivity in the picomole to high femtomole range (3,4). However, to date, the analysis of oligonucleotides by MALDI-MS has only been partially successful. This class of highly charged compounds is currently attracting much attention due to their applications as tools for molecular biology, as gene probes for diagnosis, or as potential drugs interacting on the level of nucleic acids (5). In particular the antisense technology requires modified oligonucleotides with higher *in vivo* stability and improved capability of cellular uptake. The analysis and structural verification of oligonucleotides containing the modified building blocks is often very difficult and time consuming or even impossible by conventional methods (e.g. enzymatic degradation and analysis by HPLC).

The main difficulties of MALDI-MS for the analysis of oligonucleotides seem to be on one hand the rather inefficient desorption and/or ionization behavior of larger molecules and on the other hand their high tendency to form adducts with sodium and potassium ions giving rise to multiple peaks in the spectrum for each molecular entity. This leads to broad signals from which an accurate mass determination becomes extremely difficult (6). Recently, it has been demonstrated that the addition of cation exchange resin in the ammonium form to the sample on the probe tip greatly decreases the amount of complexed alkali metal ions (7). In addition to difficulties caused by ion adducts, the length of oligonucleotides, base composition, and the choice of matrix effects the resolution and intensity of the obtained mass signals. In particular, oligothymidylates were shown to produce the most intense signals by this technique of all oligodeoxyribonucleotides investigated (3,8). The matrix compounds used in these experiments were 2,5-dihydroxybenzoic acid and 3,5-dimethoxy-4-hydroxycinnamic (sinapinic) acid. Mixtures of 2-aminobenzoic acid/nicotinic acid (7) or 3-hydroxy-4-methoxybenzaldehyde/methylsalicylic acid (9) were also demonstrated to be useful as matrices for the analysis of oligothymidylates and mixed sequences. Very recently, 3-hydroxypicolinic acid was identified as valuable matrix for the desorption and ionization of longer oligonucleotides with mixed sequences although signals with rather low resolution were obtained (10). Besides using organic carboxylic acids as matrices, data were published on the desorption of oligonucleotides up to 60–80 nucleotides in length from a frozen water surface on a corroded copper substrate (11). Using this method, the sample preparation is very elaborate and a homogeneous matrix is difficult to achieve due to the high vapor pressure of the frozen water surface under high vacuum. Furthermore the reproducibility of these experiments is reported to be poor (11). As an alternative to using new matrix compounds absorbing in the range 260–355 nm, an increase of the laser wavelength to the IR-range in combination with suitable matrix compounds can be useful (7,10). Specifically the combination

of an infrared laser with wavelengths of 2.79 μm, 2.94 μm or 10.6 μm and succinic acid as matrix was shown to be successful for the mass analysis of several oligonucleotides (7).

We have identified a new non-carboxylic acid matrix, 2,4,6-trihydroxy-acetophenone, which, in combination with diammonium sulfate, diammonium hydrogen citrate or diammonium-L-tartrate, is shown to be highly efficient for the desorption of natural and modified oligonucleotides. This methodology can be used in combination with partial digestion of oligonucleotides by 5′- and 3′-exonucleases for the sequence determination.

The commonly used methods for the sequencing of short oligonucleotides such as Maxam–Gilbert sequencing (12) or the wandering spot method (13) are very laborious and usually require the handling of radioactive material. The simple technique described herein represents a great improvement for the rapid sequencing of short oligonucleotides containing natural and modified building blocks.

RESULTS AND DISCUSSION

2,4,6-Trihydroxy acetophenone was found to be especially well suited for the desorption of oligonucleotides by MALDI MS with the additional benefit of being only slightly acidic compared to more commonly used matrices as e.g. sinapinic acid or 2,5-dihydroxy benzoic acid and therefore, allowing the desorption under milder conditions. The use is not only limited to the analysis of these highly charged compounds; also carbohydrates, proteins and metal complexes were shown to desorb well, producing intense and highly resolved signals.

The oligonucleotides 5′-d(AGCTAGCT) **I** (8-mer) and 5′-d(AGCTAGCTAGCTAGCT) **II** (16-mer) were analyzed after HPLC-purification and lyophilization by MALDI-TOF MS using 2,4,6-trihydroxy acetophenone as matrix (Figure 2). The omission of ammonium salts from the sample preparation led to multiple sodium ion adducts, the signals of which could only be resolved for the short 8-mer **I** (Figures 2a and c). For the 16-mer **II** a broad peak was obtained (Figure 2c) from which an accurate mass determination was not possible. The addition of a large excess of diammonium hydrogen citrate to the samples suppressed the alkali-ion adducts almost completely and resulted in an increase of signal intensity and resolution (Figures 2b and d). Using this sample preparation, a signal resolution (m/Δm, full width at half maximum) between 220 and 280 was routinely obtained. A plausible explanation of this observation is that the presence of a large excess of ammonium salt suppresses sodium and/or potassium ion adduct formation given that the accompanying anion of the added salt possesses sufficient high affinity for the alkali cation. Therefore only phosphodiester–ammonium ion pairs are desorbed into the vacuum where they decompose with proton transfer to give free phosphodiesters and ammonia. Because of our interest in antisense compounds, the methodology described above has been successfully applied to the analysis of oligos only up to 24 nucleosides in length, thereby not reaching an upper limit in size yet.

The molecular weights of oligonucleotides (2–24 mers) can now be routinely determined by MALDI-TOF MS using a combination of diammonium hydrogen citrate and 2,4,6-trihydroxy acetophenone as matrix with an accuracy of better than ±2 mass units (deviation <0.05%) without using an internal standard. The instrument is calibrated using a sample

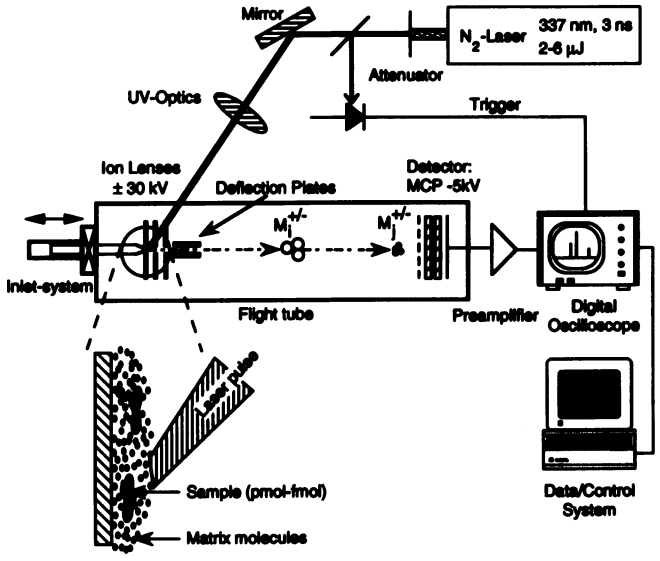


Figure 1. Illustration of the laser desorption time of flight mass spectrometer used for the analysis of oligonucleotides.

of $d(T)_{10}$ which yields two distinct peaks ($[M-H]^-$ and $[2M-H]^-$).

It was of great interest to make use of this technology in the analysis of either RNA or modified oligonucleotides for which characterization is either labor intensive and/or extremely difficult. For example the homopurine oligoribonucleotide 5′-r(AGAGAGAGAGAAAAA) **III** and the fluorescent 16-mer 5′-d(fluorescein-NH-TAAAACGACGGCCAGT) **IV** (Figure 3) have been analyzed. The oligodeoxyribonucleotide conjugate **IV** desorbed quite similarly to DNA and the attachment of one fluorescein molecule to the amino oligonucleotide could easily be verified by determination of the correct molecular weight. Oligoribonucleotides appear to desorb from 2,4,6-trihydroxy-acetophenone with greater ease than DNA oligomers and even t-RNA^{Phe} was shown to desorb, although, with a loss of precision (deviation <0.2%, *data not shown*). For the RNA 15-mer **III** a well resolved and intense peak was obtained that allowed the mass determination with high accuracy. The MALDI-TOF MS analysis of a different batch of the same RNA oligomer **III**, containing impurities as shown by capillary gel electrophoresis, clearly revealed not only the presence but also the identity of these impurities. In addition to the usual (n−1)-mer contaminant, two compounds were identified corresponding to the intact 15-mer carrying one and two *tert.*-butyl-dimethylsilyl (TBDMS) groups, respectively, that were used to protect the 2′-hydroxy function during the RNA synthesis *(data not shown)*. This example clearly reflects the power of this technology allowing not only the detection of impurities—as possible with HPLC or gel electrophoresis—but also in most cases the identity of these side products. Presently, quantitative analysis of the spectra is not possible due to the local heterogeneity of the matrix and the dependence of signal intensities on molecular weight and chemical composition of individual compounds.

In addition to the molecular weight determination, the sequence information of a particular oligonucleotide would be highly desirable. Especially oligonucleotides containing non-natural building blocks cannot be sequenced according to the standard

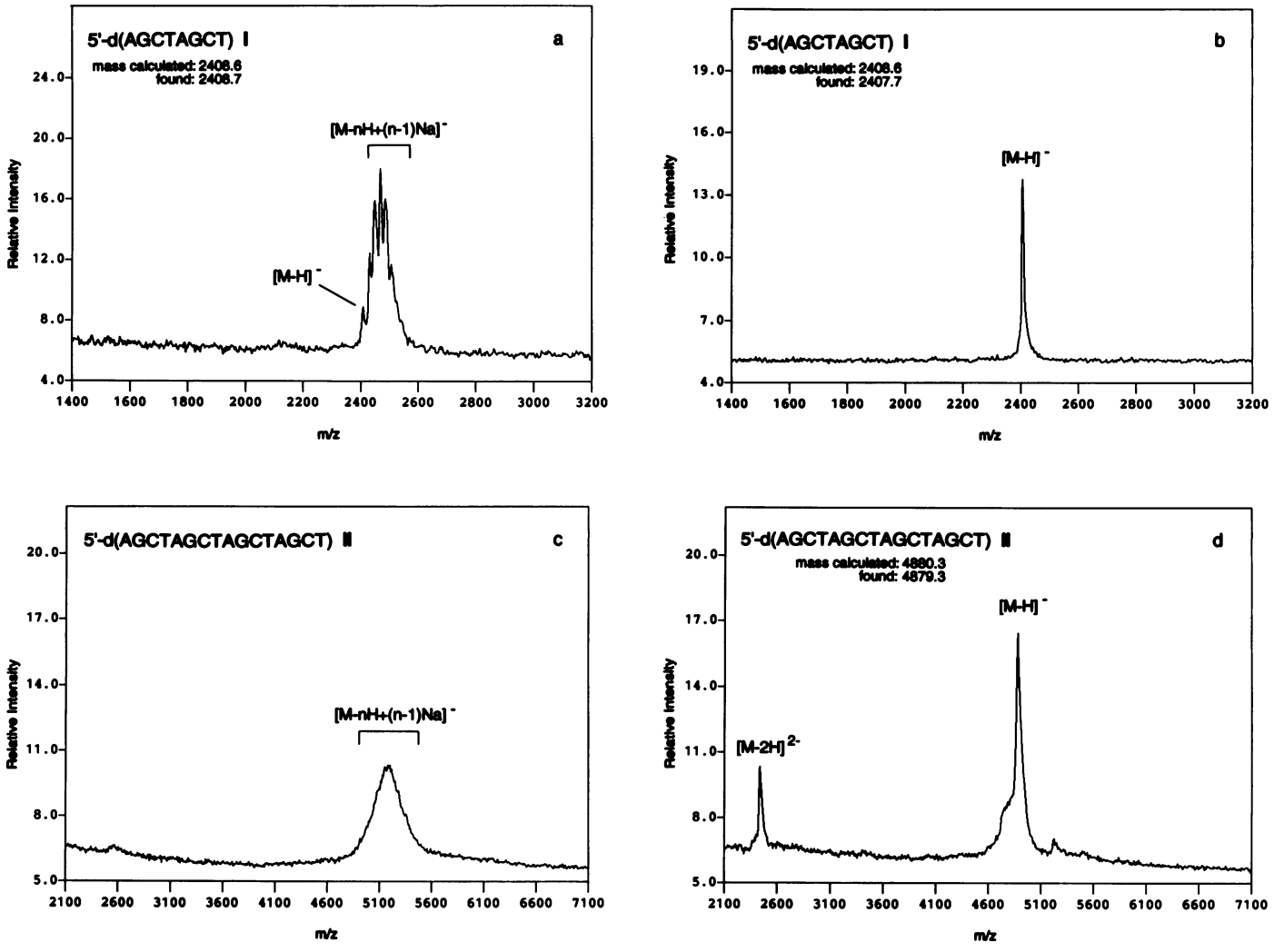
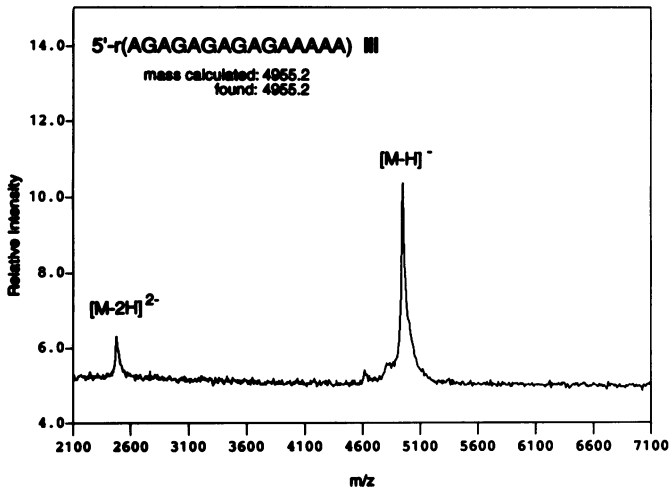


Figure 2. Negative ion MALDI-TOF MS of the oligonucleotides 5′-d(AGCTAGCT) **I** and 5′-d(AGCTAGCTAGCTAGCT) **II** with 2,4,6-trihydroxy acetophenone as matrix. Comparison without **(a, c)** and with **(b, d)** diammonium hydrogen citrate (25 mM final concentration) added to the samples.

techniques which were originally developed for DNA and RNA oligomers and are limited to those compounds (10, 11). The mass determining technology described above in combination with a time dependent degradation by 5′- and 3′-exonucleases, however, provides a straightforward and simple method to obtain the sequence information of oligonucleotides containing modified building blocks. As a model compound we chose 5′-d(GCTTXC-TCGAGT) **V**, carrying in position 5 a modified nucleoside (X = 2′-O-methyl adenosine). The oligonucleotide was digested in two separate reactions by the termini specific enzymes Calf Spleen Phosphodiesterase (CSP, 5′-exonuclease) and Snake Venom Phosphodiesterase (SVP, 3′-exonuclease). To obtain an optimal distribution of cleavage products for a particular oligonucleotide and a given batch of enzyme, aliquots were removed from the digestion reactions at time intervals and directly analyzed by MALDI-TOF MS. Such spectra from partial digestions of oligonucleotide **V** are presented in Figures 5 and 6. CSP sequentially degrades the oligonucleotide **V** from the 5′-end until it reaches the 2′-O-methyl adenosine that resists further cleavage (Figure 5). From the five distinct peaks observed, the first four nucleotides at the 5′-end of the oligonucleotide can clearly be assigned because of different individual masses for each of the four 2′-deoxyribonucleotides (the calculated mass differences are as follows: 'pdA': 313.2; 'pdC': 289.2; 'pdG': 329.2; 'pdT': 304.2). A different picture is obtained by enzymatic digestion with SVP from the opposite end (Figure 6). The enzyme degrades nucleotide by nucleotide including the modified ribonucleotide with a molecular weight corresponding to the mass difference between peak 1 and 2 (calculated: 343.2; found: 343.0). Together both spectra allow the definite sequence determination of this particular oligonucleotide as can clearly be seen from the corresponding Tables in Figures 5 and 6. This general technique described above can be applied to various types of oligonucleotides with the exception of those that are resistant towards exonucleases and those containing different nucleotides with similar molecular weights such as thymidine and 2′-deoxy-5-methylcytidine.

In summary we have demonstrated an improved methodology using MALDI-TOF MS for the analysis and sequence determination of natural and modified oligonucleotides with mixed base composition. In particular the combination of the new matrix, 2,4,6-trihydroxy acetophenone, with ammonium salts



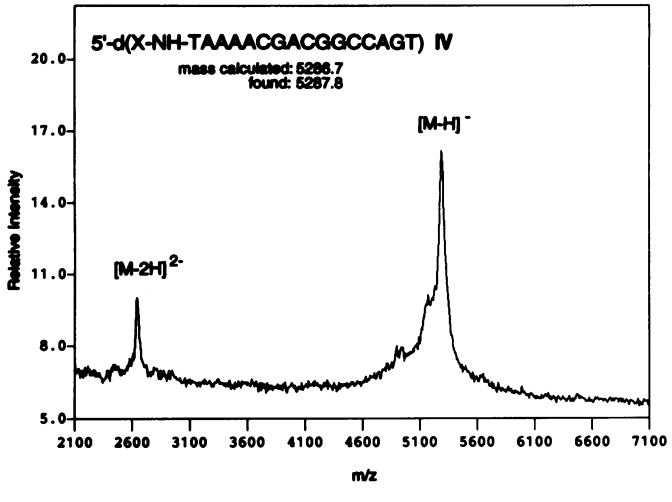


Figure 3. Molecular weight determinations by negative ion MALDI-TOF MS of the oligoribonucleotide 5'-r(AGAGAGAGAGAAAAA) III and the fluorescein labelled oligodeoxyribonucleotide 5'-d(fluorescein-NH-TAAAACGACGGCC-AGT) IV with 2,4,6-trihydroxy acetophenone as matrix and diammonium hydrogen citrate as additive.

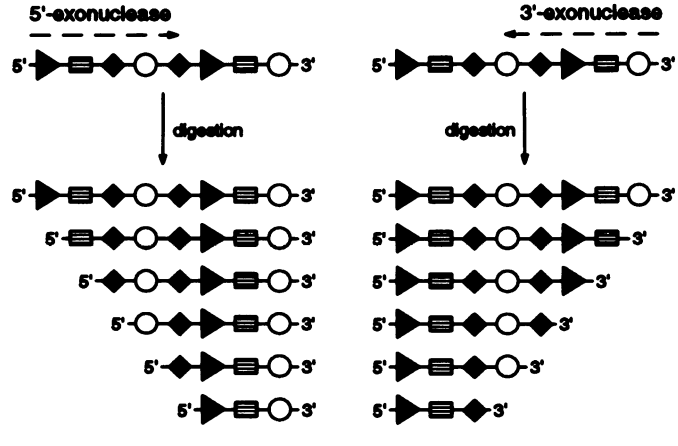
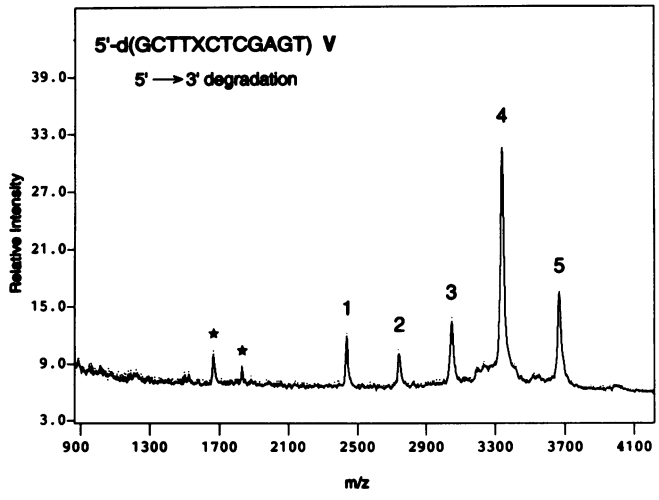


Figure 4. Schematic presentation of oligonucleotide sequencing by exonucleolytic degradation with Calf Spleen Phosphodiesterase (CSP, 5'-exonuclease) and Snake Venom Phosphodiesterase (SVP, 3'-exonuclease) followed by analysis of the resulting mixtures.



Peak	Sequence	Mass calc.	Mass found	Δm
1	5'-d(XCTCGAGT)	2438.7	*2438.3*	0.4
2	5'-d(TXCTCGAGT)	2742.9	*2742.8*	0.1
3	5'-d(TTXCTCGAGT)	3047.1	*3046.9*	0.2
4	5'-d(CTTXCTCGAGT)	3336.3	*3336.6*	-0.3
5	5'-d(GCTTXCTCGAGT)	3665.5	*3665.8*	-0.3

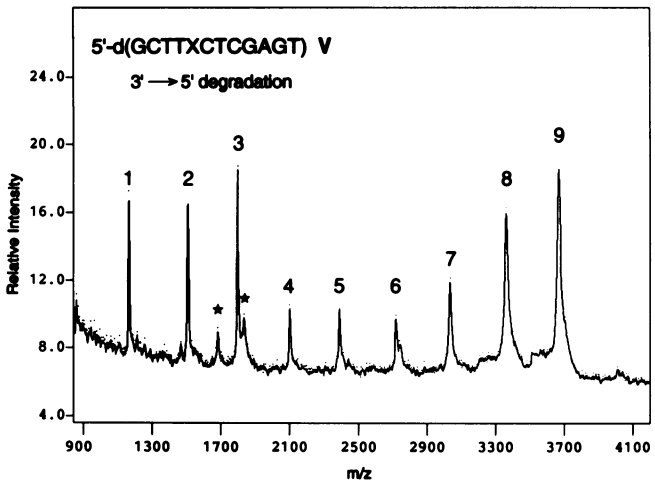
Figure 5. Negative ion MALDI-TOF MS resulting from partial digestions by CSP of the oligonucleotide 5'-d(GCTTXCTCGAGT) V carrying a modified nucleoside (X = 2'-O-methyl adenosine) in position 5. The sample was taken after 30 minutes digestion time. The peaks marked with asterisks represent doubly charged ions $[M-2H]^{2-}$ belonging to peaks # 4 and 5.

such as diammonium hydrogen citrate gives rise to well resolved and intense peaks making this technology extremely useful in such areas as nucleic acid chemistry, medicinal chemistry, diagnostics, and molecular biology. Additional attractive features of MALDI-TOF MS are ease of handling and rapid data collection and analysis. The extension of this technology to the sequencing of genomic DNA might become possible if both efficient desorption of longer oligomers (100–250 mers) and increased sensitivity to the low femtomole range can be achieved.

EXPERIMENTAL

Oligonucleotide synthesis and purification

Oligodeoxyribonucleotides were synthesized on an automated DNA synthesizer ABI 394B (Applied Biosystems Foster City, USA) using β-cyanoethyl phosphoramidites (Glen Research USA) and standard synthesis protocols (14). The oligonucleotides were purified by reversed phase HPLC with the 4,4'-dimethoxytrityl (DMT) group on, using a RPC_{18} column (4.6 mm×250 mm, ODS Hypersil Shandon Runcorn, GB) eluting with a linear gradient from 15% to 45% B in 45 minutes (eluent A: 50 mM triethylammonium acetate pH 7; Eluent B: 50 mM triethylammonium acetate pH 7 in 70% acetonitrile). After removal of the remaining DMT group with 80% acetic acid and extraction with diethylether, the oligonucleotides were lyophilized



Peak	Sequence	Mass calc.	Mass found	Δm
1	5'-d(GCTT)	1163.8	*1163.9*	-0.1
2	5'-d(GCTTX)	1507.1	*1506.9*	0.2
3	5'-d(GCTTXC)	1796.2	*1796.2*	0
4	5'-d(GCTTXCT)	2100.4	*2100.5*	-0.1
5	5'-d(GCTTXCTC)	2389.6	*2389.8*	-0.2
6	5'-d(GCTTXCTCG)	2718.9	*2719.3*	-0.4
7	5'-d(GCTTXCTCGA)	3032.1	*3032.8*	-0.7
8	5'-d(GCTTXCTCGAG)	3361.3	*3362.0*	-0.7
9	5'-d(GCTTXCTCGAGT)	3665.5	*3666.0*	-0.5

Figure 6. Negative ion MALDI-TOF MS resulting from partial digestions by SVP of the oligonucleotide 5'-d(GCTTXCTCGAGT) V carrying in position 5 a modified nucleoside (X = 2'-O-methyl adenosine). The sample was taken after 30 min digestion time. The peaks marked with asterisks represent doubly charged ions $[M-2H]^{2-}$ belonging to peaks # 8 and 9.

twice from 50% aqueous ethanol to remove residual triethylammonium acetate.

The 8 mer oligonucleotide d(AGCTAGCT) was purified by thin layer chromatography (15) (TLC plates with fluorescent indicator silica gel 60_{F254}, Merck Darmstadt, Germany) using isopropyl alcohol/water/aq. ammonia (25%) 11:7:2 as eluent. The silica gel containing the oligonucleotides was scratched from the glass plates and eluted by the addition of three times 500 μl of 70% aqueous ethanol. The supernatant was removed after centrifugation and evaporated to dryness. The resulting oligonucleotide was redissolved in sterile water and used for UV measurements and mass spectrometry.

RNA was synthesized using 2'-O-TBDMS protected, β-cyanoethyl phosphoramidites (Millipore, USA) and standard reaction protocols (16). After deprotection with a saturated solution of ammonia in dry methanol at 60°C for 8 hours and treatment of the residual oligonucleotide with a freshly prepared solution of tetrabutylammonium fluoride in dry THF for 16 hours at room temperature, the oligonucleotide was passed through a sep-pak cartridge (Waters corp., USA) to remove the remaining fluoride salt and purified by gel electrophoresis. The oligonucleotide containing gel slices were cut out and eluted with 0.5 M ammonium acetate over night at 4°C. After dialysis against water the oligonucleotide was used directly for the mass measurements (all aqueous buffers and solutions were pretreated with diethyl pyrocarbonate to remove possible RNase contaminants).

5'-d(Fluorescein-NH-TAAAACGACGGCCAGT) **IV** was prepared by incorporation of monomethoxy trityl protected 5'-aminothymidine as described (17). The detritylated amino oligonucleotide (0.5 μmole synthesis) was coupled with fluorescein isothiocyanate (50 μmole, Isomer I, Fluka) in 0.25 M sodium carbonate buffer pH 9.0 in DMF/water 1:1 directly on the solid support for 21 hours. After washing the solid support with DMF, water, methanol and acetonitrile to remove excess reagents, the oligonucleotide was removed from the solid support and deprotected with aqueous ammonia (32%, 18 h at 37°C) and purified by gel electrophoresis. The fluorescent band was cut out from the gel and electroeluted (Biotrap, Schleicher & Schüll) in 50 mM TBE-buffer (18).

Mass spectrometry of oligonucleotides

Mass spectra were run on an LDI 1700 (Linear Scientific Inc., Reno, USA). The average power of the nitrogen laser (337 nm) used in the experiments was about 10^6 W/cm^2 (4–10 μJ/pulse). All measurements were performed using the negative detection mode. The spectra were obtained by overlaying 10–50 single laser pulses. The spectrometer was calibrated externally with the $[M-H]^-$ and $[2M-H]^-$ mass peaks of dT_{10} as reference.

Sample preparation

10 μl of an 0.5 M solution of 2,4,6 trihydroxy acetophenone in ethanol, 5 μl of a 0.1 M aqueous solution of diammonium hydrogen citrate or diammonium-L-tartrate were mixed. 1μl of an oligonucleotide containing solution (5–10 OD/ml, 150–300 μg/ml) was added and the mixture was briefly vortexed. 1 μl of this solution was applied to the probe tip and the solvents gently removed in vacuum.

Sequencing of oligonucleotides

The sequencing of oligonucleotides was carried out by time dependent enzymatic degradation reactions. All reactions were run in water without the addition of salts or buffer. Oligonucleotides were degraded from both ends in separate reactions.

5'-3' degradation: 1 μl of Calf Spleen Phosphodiesterase (CSP, EC 3.1.16.1, Boehringer Mannheim, Germany; 10^{-3} u/μl) was added to 20 μl of an oligonucleotide containing solution (5–10 OD/ml, 150–300 μg/ml) and the mixture was incubated at 37°C. Samples of 1 μl were taken every 15 minutes and analyzed as described above.

3'-5' degradation: 1 μl of Snake Venom Phosphodiesterase (SVP, EC 3.1.15.1, Boehringer Mannheim, Germany; 2×10^{-3} u/μl) was added to 20 μl of an oligonucleotide containing solution (5–10 OD/ml, 150–300 μg/ml) and the mixture was incubated at 37°C. Samples of 1 μl were taken every 15 minutes and analysed as described above.

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