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Matrix-assisted Laser Desorption Mass Spectrometry of Homopolymer Oligodeoxyribonucleotides. Influence of Base Composition on the Mass Spectrometric Response

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Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has the potential for providing a rapid alternative to gel electrophoresis for DNA sequence analysis provided that an intense mass spectrometric response can be obtained from mixtures of DNA fragments containing up to 300 nucleotides. MALDI-MS has not yet proved viable for such analyses because the MS response falls off rapidly for mixed-base DNA fragments containing more than 20–30 nucleotides. Previous studies have demonstrated that base composition is a critical factor in the MALDI-MS response of oligodeoxyribonucleotides. This paper describes an investigation of the physical roots of the observed influence of base composition on the mass spectrometric response, focusing on homopolymer oligodeoxyribonucleotides (dT_7, dT_{10}, dT_{18}, dT_{36}, dG_7, dG_{10}, dG_{18}, dI_{18} and dU_{18}) and dT_5G_5. Forty-eight different matrix compounds were tested for their ability to produce laser desorption masses spectra from such homopolymer oligodeoxyribonucleotides. Considerably stronger mass spectrometric responses were obtained from polydeoxythymidines than from polydeoxyguanosines, polydeoxycytidines and polydeoxyadenosines. Although mass spectral peaks corresponding to dT_{18} were observed from 20 of the matrices studied, no discernible response was observed for dG_{18} from any of these matrices. To elucidate the physical basis for origins of the observed differences in response, a number of factors were investigated including the ionization efficiency, the tendency towards fragmentation and the extent to which the oligodeoxyribonucleotides were incorporated into the matrix crystals. The results of these experiments indicate that low ionization efficiency is not a likely main contributor to the low response to polydeoxyguanosines, fragmentation is a likely main contributor to the low response to polydeoxyguanosines, the overall incorporation of polydeoxyguanosines into matrix crystals is comparable to that for polydeoxythymidines and the exocyclic amino group of guanosine, adenosine and cytidine has a strong inhibitory effect on the mass spectrometric response.

INTRODUCTION

DNA sequence determination is normally carried out by generating a set of DNA fragments by the Sanger[1] or Maxam and Gilbert method[2] and requires the separation of individual fragments differing by one nucleotide in length. To date only a single technique, high-resolution gel electrophoresis, has proved capable of providing the requisite resolving power over a useful range of fragment lengths (~300 nucleotides).[3] Matrix-assisted laser desorption/ionization mass spectrometry (MALDI)[4,5] is a new technique that allows the rapid, accurate analysis of proteins in the relative molecular mass range extending up to a few hundred thousand. Provided that the method can be extended, with similar success, to the analysis of DNA fragments, then MALDI has the potential for providing a rapid alternative to gel electrophoresis for DNA sequence analysis.

The possibility of using MALDI for DNA sequence analysis has stimulated a number of recent studies[6–20] that incorporate two different approaches for producing ions from DNA fragments. The first involves ablation by laser irradiation of a thin, frozen layer of water containing the oligonucleotide molecules of interest.[6,17] In this method, the laser light energy is initially deposited on the surface of a metallic substrate on which the frozen sample is placed. Mass spectra were obtained of oligonucleotides that include a double-strand 28mer[6] and polynucleotide mixtures up to 60 nucleotides in length.[17] The mass spectra exhibited good signal-to-noise ratios and little evidence of fragmentation, but the appropriate experimental conditions were difficult to reproduce.[17] The second approach involves the desorption and ionization of oligonucleotide molecules incorporated into a solid matrix of small organic compounds.[4,5] Efforts in this area have concentrated on a search for matrix compounds that are suitable for the efficient volatilization and ionization of oligonucleotides and on improvements in the methodology of sample preparation and purification. Early reports include the observation of mass spectra of 5S-rRNA (RMM = 38 000),[7] a series of tetramers[8] and oligonucleotides ranging in length between 2 and 6 nucleotides.[9] All of these results were obtained from a matrix of nicotinic acid. Tang *et al.*[12] obtained mass spectra of mixtures of oligonucleotides ranging up to 34 nucleotides in length using a mixture of 3-methylsalicylic acid and 3-hydroxy-4-methoxybenzaldehyde. Currie and Yates[15] extended the workable mass range of certain

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matrices by the addition of volatile ammonium salts to the sample–matrix mixture and obtained negative-ion mass spectra of a 60mer composed of repetitive dGCAT stretches from 2,5-dihydroxybenzoic acid (for the sake of simplicity, oligodeoxyribonucleotides are referred to as oligonucleotides, and are designated in the 5′–3′ direction by line formulae, such as dACGT, where dA = 2′-deoxyadenosine, dG = 2′-deoxyguanosine, and so forth). Nordhoff *et al.*[18] compared the use of ultraviolet and infrared laser sources for a number of different matrices. Although the mass spectra obtained with IR-wavelength irradiation (2.94 μm) were more difficult to obtain than with the UV irradiation (337 nm), it proved possible to obtain higher quality spectra of the larger oligonucleotides with IR irradiation. This group also found that removal of alkali metal cations from the sample significantly improved the mass spectrometric response. Wu *et al.*[20] reported the discovery of a matrix compound, 3-hydroxypicolinic acid, which yields improved spectra of oligonucleotides compared with most previously reported matrices.

Although the above studies reflect significant progress in the analysis of oligonucleotides by MALDI, considerable further improvement of the method is needed before the technique can be usefully applied to DNA sequence analysis. Thus, while spectra of oligonucleotides up to a 67mer have been reported,[20] the mass spectrometric response has been found to fall off precipitously for mixed-based DNA fragments containing more than 20–30 nucleotides.[12,20] To investigate the effect of base composition on the mass spectrometric response, Parr *et al.*[13] studied a series of homopolymer oligonucleotides. Whereas intense spectra were observed for dT_{10}, the spectra of dC_{10}, dA_{10}, and dG_{10} were either weak or absent, indicating that base composition is a critical factor in the mass spectrometric response of oligonucleotides.[13] Several other MALDI measurements have been reported of thymidine homopolymers, including mixtures of dT_{12-18},[10,11] dT_{12-30}[13] and dT_n mixtures ranging from 20 to 100 nucleotides in length.[19] For other homopolymers, only a few mass spectra with weak signal intensities have been published.[6,13]

This study was undertaken with the belief that an understanding of the physical basis for the observed influence of base composition on the mass spectrometric response may lead to rational means for a general improvement of the MALDI mass spectrometric response to oligonucleotides. In particular, we evaluated the influence of base composition on the laser desorption mass spectrometric response of the homopolymer oligonucleotides (dT_7, dT_{10}, dT_{18}, dT_{36}, dG_7, dG_{10}, dG_{18}, dC_{18}, dA_{18}, dI_{18} and dU_{18}) and dT_5G_5, and the physical factors that influence this response. Forty-eight different matrix compounds were tested for their ability to produce laser desorption mass spectra from such homopolymer oligonucleotides. In agreement with previous studies,[13] we found that considerably stronger mass spectrometric responses were obtained from polydeoxythymidines than from polydeoxyguanosines, polydeoxycytidines and polydeoxyadenosines. Indeed, mass spectral peaks corresponding to dT_{18} were observed from 20 of the matrices studied, whereas no discernable response was observed for dG_{18} from any of these matrices. In an effort to elucidate the physical basis for the observed differences in response, a number of factors were investigated including the ionization efficiency, the tendency towards fragmentation and the extent to which the oligonucleotides were incorporated into the matrix crystals.

EXPERIMENTAL

The oligodeoxyribonucleotides dA_{18}, dC_{18}, dT_{18}, dG_{18}, dI_{18} and dU_{18} were obtained as triethylammonium salts from New England Biolabs (Beverly, MA, USA) and used without purification. Oligodeoxyribonucleotides dG_7, dT_7 and a second sample of dG_{18} were synthesized in the Protein Sequencing Facility at Rockefeller University. dG_{10}, dT_5G_5 and dT_{10} were obtained from National Biosciences (Plymouth, MN, USA). Concentrated solutions (200 μM) were prepared by dissolution in high-purity water (Burdick and Jackson Laboratories, Muskegon, MI, USA) and were stored frozen at −20 °C. The samples of dT_{18} and dG_{18} were analyzed periodically during the course of the present experiments by reversed-phase high-performance liquid chromatography (HPLC) to check for the occurrence of degradation reactions during storage.

Matrix compounds were obtained from several sources. Caffeic acid, ferulic acid, 4-amino-2,3,5,6-tetrafluorobenzoic acid, 3-aminopyrazine-2-carboxylic acid, 4-hydroxy-α-cyanocinnamic acid, 4-nitrocinnamic acid, α-methylcinnamic acid, 3-nitrocinnamic acid, 3-dimethylaminobenzoic acid, *trans*-3-(3-pyridyl)acrylic acid, guanine, rhodamine 6G, carminic acid, rhodamine B, adenine, 4-aminobenzamide, coumarin-3-carboxylic acid, esculetin monohydrate and thymine were obtained from Aldrich (Milwaukee, WI, USA), 2,5-dihydroxybenzoic acid, 3,4-dimethoxybenzoic acid, vanillic acid, psoralen, thiourea, 6-hydroxynicotinic acid, 2-thiouracil, uracil, β-naphthoic acid, adenosine monophosphate, janus green, azathiopurine, riboflavin, ethidium bromide, 1,N^6-ethenoadenine, urocanic acid and acridine orange from Sigma Chemical (St. Louis, MO, USA), α-cyanocinnamic acid, esculetin, 4-dimethylaminocinnamic acid, α-ethyl-*m*-nitrocinnamic acid, 4-acetoxycinnamic acid, 4-bromocinnamic acid and 4-chlorocinnamic acid from Lancaster (Windham, NH, USA), 3-indole-3-acrylic acid from Fluka (Buchs, Switzerland) and chloranilic acid from United States Biochemical (Cleveland, OH, USA). Substance P, angiotensin III, bovine insulin, oxidized bovine insulin A- and B-chain and bovine cytochrome *c* were used for calibration and were obtained from Sigma Chemical.

The spectra were obtained on a linear time-of-flight laser desorption mass spectrometer constructed at Rockefeller University and described elsewhere.[21,22] Laser pulses were produced in a Q-switched Lumonics HY 400 neodymium/yttrium aluminum garnet laser (266 or 355 nm, 10 ns duration) and focused to a ellipse (0.1 and 0.3 mm short and long axes) by a lens (12 in focal length) through a fused-silica window on to the sample inside the mass spectrometer. The desorbed ions were accelerated by a static electric potential of −20 kV

for negative ions and +30 kV for positive ions. The ions passed through a 2 m flight tube containing an electrostatic particle guide[23] before reaching a detector consisting of a microchannel plate followed by a gridded discrete dynode electron multiplier.[24]

Matrix-assisted laser desorption mass spectra frequently contain peaks arising from intense fluxes of ions with masses below 500 u. This intense ion flux can easily saturate the hybrid ion detector. To avoid saturation effects, the detector is selectively switched off during the time period during which the intense flux of low-mass ions arrive at the detector. The switching is achieved by the application of a high voltage pulse to the microchannel plate. Signals were recorded using a Lecroy TR8828D transient recorder. Two hundred individual time-of-flight spectra were summed together for each compound studied and converted into a mass spectrum.

Oligonucleotides were added to saturated solutions of the matrix compounds in 30–50% acetonitrile in water to give a final oligonucleotide concentration of 2 μM. A volume of 0.5 μl of this solution was loaded on to the mass spectrometer sample probe tip (2 mm diameter), dried with forced air and immediately inserted into the mass spectrometer and analysed. Samples were washed by dipping the probe tip loaded with vacuum-dried sample into ice-cold water for 10–15 s.

The incorporation experiments were performed as follows: 16 nmol of acridine orange were added to three different vials each containing 400 μl of saturated sinapinic acid solution (35 °C) in water–acetonitrile (2 : 1, v/v). To the first vial no further solutes were added, to the second vial 800 pmol of dG_{18} were added and to the third vial 800 pmol of dT_{18} were added. After vortex mixing, the solutions were transferred to a glass plate containing three 1 cm^3 capacity hollows and left overnight (at +4 °C) in a box in which the exchange of air was limited. The resulting crystals were washed three times with cold water and dried at room temperature. For the HPLC determination of the extent of oligonucleotide incorporation into the resulting matrix crystals, the colored crystals containing dT_{18} and dG_{18} were each weighed and dissolved in 20% aqueous acetonitrile. Measured aliquots were then injected into the HPLC system and analysed by measuring the absorption at 260 nm. The absorption was normalized to standard oligonucleotide solutions of comparable concentrations. The chromatography was performed using a 1.0 mm diameter Vydac C_4 reversed-phase column on an Ultrafast Microprotein Analyzer (Michrom BioResources, Pleasanton, CA, USA) under the following conditions: buffer A, 90% 10 mM triethylammonium acetate (pH 5.8)–10% acetonitrile; buffer B, 10% 10 mM triethylammonium acetate (pH 5.8)–90% acetonitrile; gradient 0–0.04 min 95% A, 0.04–12 min 95–50% A, 12–13 min 50% A, 13–15 min 50–95% A, 15–18 min 95% A.

RESULTS AND DISCUSSION

Search for effective laser desorption matrices

The objective of this part of the study was to evaluate a range of protein matrix materials for their usefulness in matrix-assisted laser desorption mass spectrometric analysis of oligonucleotides. dT_{18} was chosen as a test compound because oligothymidines have previously been shown to produce intense responses by MALDI[10,11,13] and because we assumed that a matrix would not be generally useful if it does not yield a discernable response for dT_{18}. Conversely, if the matrix under study was found to produce a significant signal of intact dT_{18}, the matrix was tested for its effectiveness in producing ions signals from the other oligonucleotides.

Table 1. Compounds tested as matrices for MALDI of dT_{18}

Strong response	
Caffeic acid	2,5-Dihydroxybenzoic acid
Ferulic acid	4-Hydroxy-α-cyanocinnamic acid
3-Indole-3-acrylic acid	Sinapinic acid
Vanillic acid[a]	4-amino-2,3,5,6-tetrafluorobenzoic acid[a]
3-aminopyrazine-2-carboxylic acid	3,4-Dimethoxybenzoic acid[a]
α-Cyanocinnamic acid	Esculetin
Psoralen	Thiourea[a]
Weak response	
4-Dimethylaminocinnamic acid	6-Hydroxynicotinic acid[a]
4-Nitrocinnamic acid	2-Thiouracil
thymine[a]	Uracil[a]
No response	
α-Ethyl-*m*-nitrocinnamic acid	2-Hydroxy-α-cyanocinnamic acid
α-Methylcinnamic acid	4-Bromocinnamic acid
4-Acetoxycinnamic acid	4-Chlorocinnamic acid
3-Nitrocinnamic acid	Chloranilic acid[a]
Urocanic acid[a]	β-Naphthoic acid[a]
Coumarin-3-carboxylic acid[a]	3-Dimethylamino benzoic acid[a]
4-Aminobenzamide[a]	*trans*-3-(3-Pyridyl)acrylic acid[a]
Guanine[a]	Adenine[a]
Adenosine monophosphate[a]	$1,N_6$-Ethenoadenine
Janus green	Azathiopurine[a]
Rhodamine 6G	Rhodamine B
Riboflavin	Carminic acid
Ethidium bromide	Acridine orange

[a] Tested at 266 nm.

At the outset of the study, we compared positive versus negative ion production for dT_{18} from ferulic acid, sinapinic acid and gentisic acid. Although intense signals from dT_{18} were observed in both polarities from all three matrices, and negative ion signals were more intense and the molecule-ion peaks were narrower than the corresponding positive ion peaks. Therefore, all further studies were carried out in the negative ion mode.

A total of 48 potential matrix compounds were screened for their ability to produce molecule-ions from dT_{18} (Table 1). A laser wavelength of either 355 or 266 nm was employed depending on the absorption characteristics of each matrix compound. Twenty of the matrices tested yielded discernible signals of intact dT_{18}. Several of these matrices have not previously been described for use with MALDI. Although the most intense and reproducible responses were obtained from ferulic acid at 355 nm and vanillic acid at 266 nm, 14 of the matrices listed in Table 1 gave relatively strong responses. Spectra obtained from three of these matrices

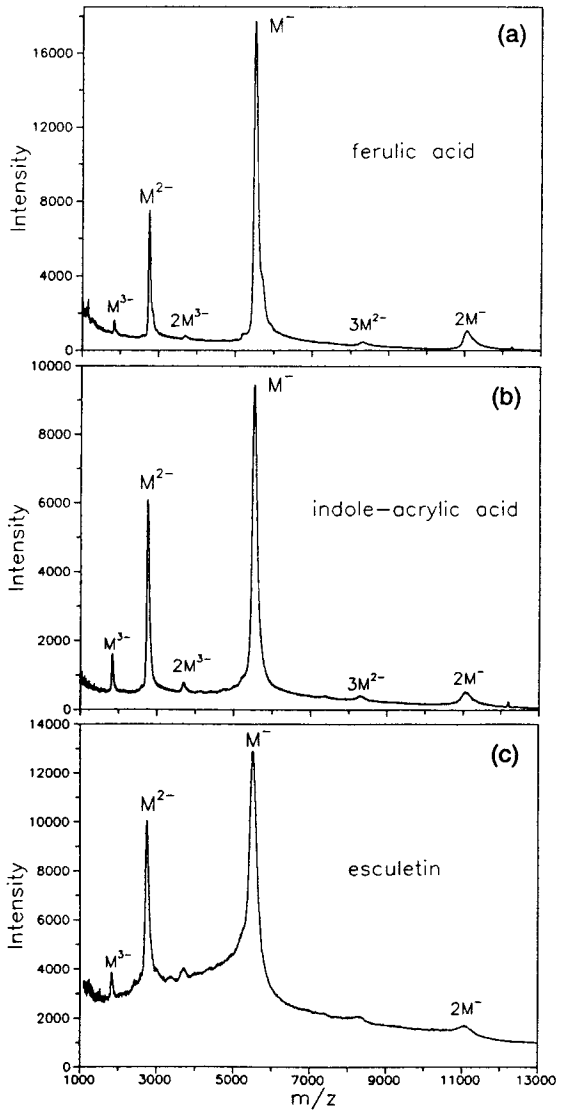

Figure 1. Negative-ion mass spectra of dT_{18} from a matrix of (a) ferulic acid, (b) 3-indole-3-acrylic acid and (c) esculetin.

(ferulic acid, 3-indole-3-acrylic acid and esculetin) are compared in Fig. 1. Each spectrum was obtained from a total loading of 1 pmol of dT_{18} on to the probe tip. The spectra show dominant singly charged ion peaks in addition to less intense peaks resulting from doubly charged ions and polymeric ion species (mostly dimers). No significant differences were observed in the main features of the spectra obtained from the same matrices at 266 versus 355 nm.

The coumarin derivative psoralen, which is widely used as a cross-linking reagent, yielded ions of dT_{18} of high intensity. Although photochemically generated polymers consisting of up to 30 psoralen monomers were observed, there was no evidence for cross-linking of the oligothymidine moieties. The neutral character of psoralen also allowed the collection of mass spectra of dT_{18} prepared for a solution with a pH value higher than is normally employed in MALDI.[25,26] Since the negative ions probably arise by deprotonation of the oligonucleotide, it may be predicted that improved ion yields can be obtained from solutions prepared at elevated pH. The present results did not support this hypothesis in that the spectra obtained from samples prepared at high pH were less intense than those obtained from samples prepared at low pH.

Mass calibration

Mass calibration was performed by adding internal calibrants to the matrix/sample solution. Attempts to use proteins such as cytochrome *c* and bovine insulin as mass calibrants were unsuccessful because the presence of oligonucleotide caused strong suppression of the signal from the protein. We do not understand the origin of the suppression effect. It may indicate a non-specific interaction between protein and DNA molecules which interferes with the mass spectrometric analysis. Parr *et al.*[13] have previously described a 'deleterious' effect of proteins on the mass spectrometric analysis of oligonucleotides. In contrast to our findings with proteins, we observed that mixtures of peptides, such as oxidized bovine insulin A- and B-chain, could be successfully employed as internal mass calibrants for oligonucleotides. To decrease the large amount of sodium adduct formation observed for the acidic A-chain, it was necessary to wash the sample on the probe tip with cold acidified water prior to the mass spectrometric analysis.[27] Mass accuracies of better than 1 part in 1000 were achieved with this procedure, as demonstrated in the mass spectrum of the 10mer dT_5G_5 (calculated molecular mass = 3106 u) obtained from a matrix of α-cyanocinnamic acid (Fig. 2). The most intense peaks in the molecule-ion region could be identified as arising from the $[M + Na - 2H]^-$ and $[M + 2Na - 3H]^-$ ions. The species with *m/z* 2976 is probably an impurity resulting from an apurination reaction, i.e. the hydrolytic loss of one guanine from the sugar-phosphate backbone. It is important to note that the effective mass accuracy decreases at higher mass where the sodium adduct peaks can no longer be resolved.

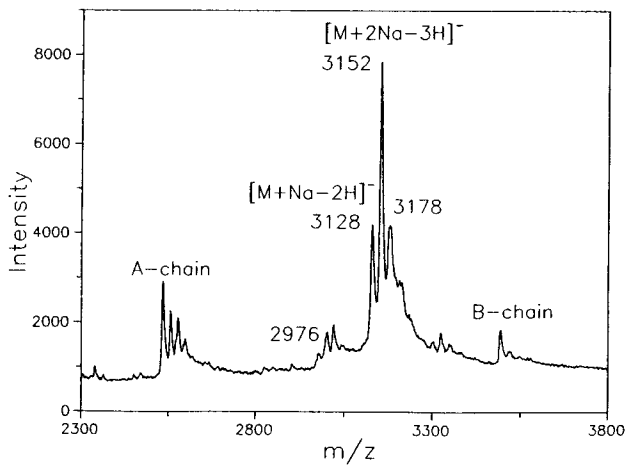

Figure 2. Expanded portion of the negative-ion mass spectrum of dT_5G_5 from ferulic acid. Oxidized insulin A- and B-chain were added as internal calibrants.

Adduct ion formation

In the mass spectrum of dT_5G_5 (Fig. 2), a number of sodium adduct ions were resolved and assigned. Similar alkali metal adduction has been observed previously in the MALDI spectra of short-chain oligonucleotides.[9,10,18] For longer-chain oligonucleotides, it becomes increasingly difficult to resolve adduct peaks and therefore to characterize the nature and amount of the observed adduction. For example, at least eight different peaks are present in the molecule-ion region of dT_{18} obtained from α-cyanocinnamic acid (Fig. 3). The weak signal seen at *m/z* 5410 may correspond to deprotonated intact dT_{18} (calculated *m/z* 5413). Electrospray ionization mass spectrometry of the dT_{18} sample yielded a molecular mass of 5414 u confirming the integrity of the sample [the electrospray mass spectrum of dT_{18} was obtained on a Sciex AP13 instrument by Dr P. F. Nielsen (Novo Nordisk, Bagsvaerd, Denmark)]. In the laser desorption mass spectrum, the most abundant ion is observed at *m/z* 5536, 126 u higher than the peak at *m/z* 5410 ascribed to the deprotonated intact molecule. The origin of the abundant adduct is not understood, but intense adduct peaks with similar mass shifts observed for dT_{18} from ferulic and sinapinic acids indicate that the additional mass probably was not due to photochemical matrix adduct formation.[21] The peak at *m/z* 5709 can be rationalized by adduction of a matrix molecule (molecular mass = 173 u) to the ion with *m/z* 5536.

The resolution of the spectrum obtained from α-cyanocinnamic acid (Fig. 3) is in the range 250–300 FWHM, which is comparable to that normally achieved for proteins with our instrument and about a factor of two higher than that obtained from most of the other matrices that we tested. This resolution is still inadequate, however, for resolving adduct ions on larger oligonucleotides (longer than 20–30 bases), leading to an effective peak broadening and further complications in mass determination.

Mass range and sensitivity

A number of investigations have been carried out on the MALDI mass spectrometric response to random-sequence oligonucleotides as a function of molecular mass.[12,17,20] In general, the signal intensities are observed to drop sharply and the molecule-ion peaks to broaden markedly for polymers containing more than 20–30 bases. This behaviour was not apparent for oligothymidines, as is evident from the spectrum of dT_{36} shown in Fig. 4(a), where very intense singly and doubly charged molecule-ions were observed from 1 pmol of sample. Also present in the mass spectrum were peaks arising from the dimer, trimer, tetramer and pentamer, reflecting a measurable range of up to 180 bases for oligothymidines. The sensitivity for dT_{36} was probed by a tenfold dilution of the sample. Signals from a total of 100 fmol loaded on to the probe tip yielded the spectrum shown in Fig. 4(b). In this latter spectrum, no intense multimer ion peaks were observed and the peak intensity shifted towards the more highly deprotonated species. Similar shifts have previously been observed as a function of sample concentration in the mass spectra of proteins observed by plasma desorption mass spectrometry,[28] keV energy secondary ion mass spectrometry[29] and matrix-assisted laser desorption mass spectrometry.[30] The effect has been rationalized as arising from a diminution of competition for charge between the different ion species as the sample concentration is decreased.

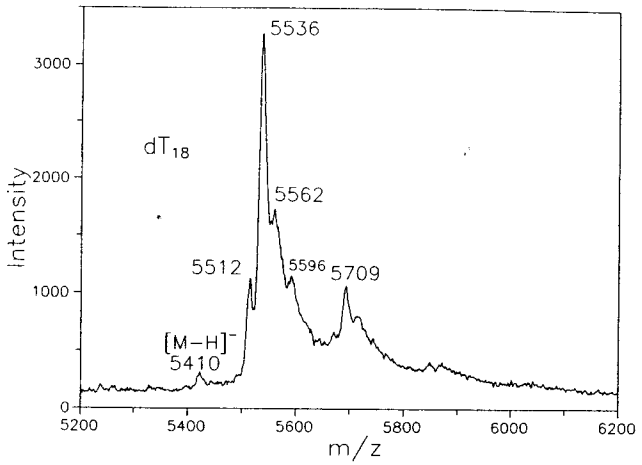

Figure 3. Expanded portion of the negative ion mass spectrum of dT_{18} from α-cyanocinnamic acid.

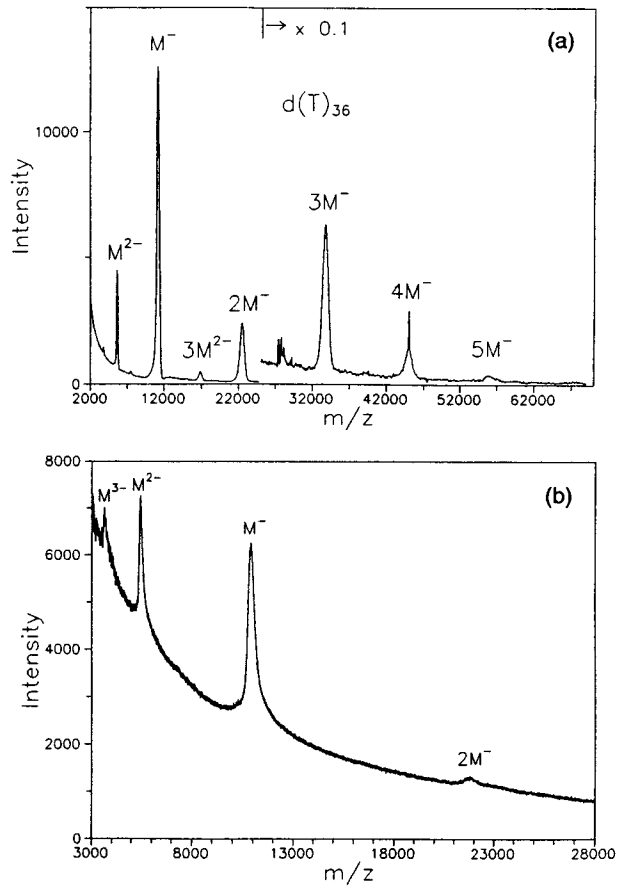

Figure 4. Negative-ion mass spectrum of dT_{36} from ferulic acid. Total sample loaded on the probe tip: (a) 1 pmol and (b) 100 fmol.

Influence of base composition

To investigate the influence of base composition on the MALDI mass spectrometric response, all of the matrices listed in Table 1 that produced discernible spectra from dT_{18} were tested for their ability to produce spectra from dG_{18}. As an example, Fig. 5 compares the mass spectrum acquired from a sample of 1 pmol of dT_{18} in caffeic acid [Fig. 5(a)] with that obtained from dG_{18} [Fig. 5(b)] under identical conditions. Whereas strong signals were observed for dT_{18}, no ions corresponding to dG_{18} were present in the mass spectrum. Similarly, none of the remaining 19 matrices that were successfully employed for dT_{18} produced an observable mass spectrometric response from dG_{18}. Attempts to analyse dC_{18} and dA_{18} in ferulic acid also did not yield any useful mass spectrometric response. These results strongly confirm the earlier observation of Parr *et al.*[13] that base composition is a critical factor for the analysis of oligonucleotides by MALDI. In addition, our results demonstrate that such effects of base composition are consistently observed for a wide variety of structurally different matrix compounds.

We hypothesized three possible causes for the lack of mass spectrometric response for polyguanosines in contrast to the strong response for polythymidines: low ionization efficiency of dG_{18}; inadequate or inappropriate incorporation of dG_{18} into the matrix crystals; and facile and extensive fragmentation of dG_{18} in the mass spectrometer. Each of these possibilities will be considered below.

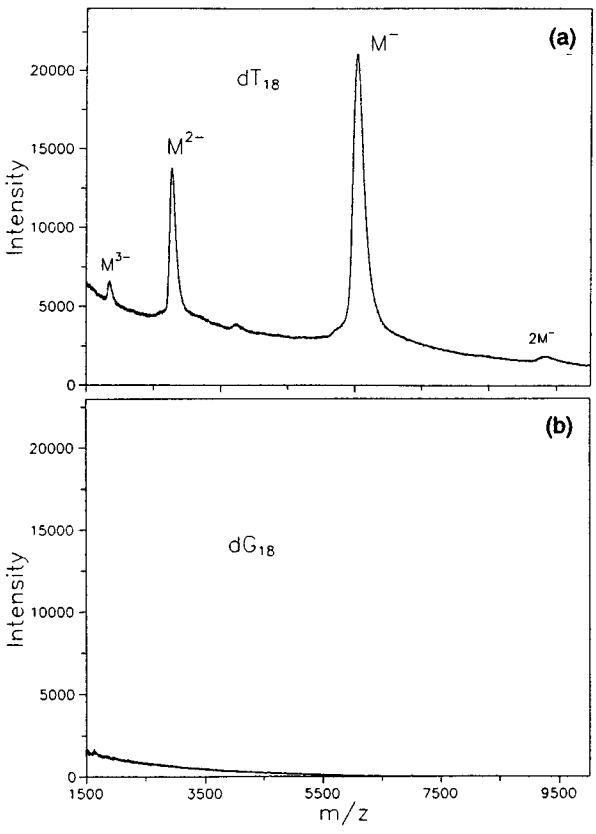

Figure 5. Negative-ion mass spectra of (a) dT_{18} and (b) dG_{18} from caffeic acid.

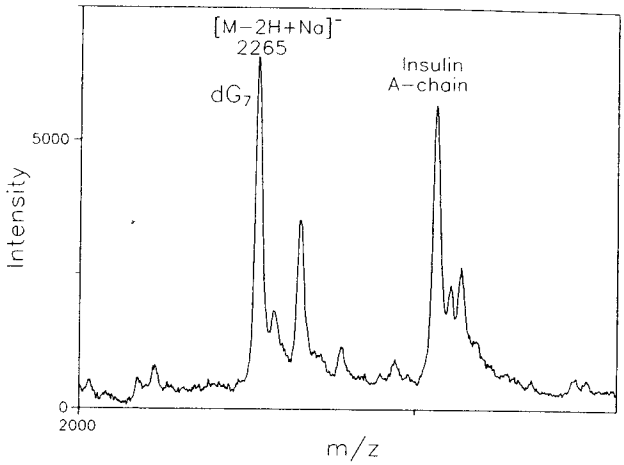

Figure 6. Negative-ion mass spectrum of dG_7 from sinapinic acid.

Ionization. Measurements obtained on a short stretch of polyguanosine (dG_7) gave a reasonably intense mass spectrometric response (Fig 6), indicating that an inherently low efficiency for ionizing polyguanosine is not the primary reason for our failure to observe longer stretches of the polymer.

Self-association and incorporation into the matrix. MALDI of proteins from solid matrix microcrystals has been demonstrated to require that the proteins of interest be incorporated into the bulk of the matrix crystals.[31,32] It appears reasonable to assume that similar bulk incorporation is required for the successful production of oligonucleotide ions by MALDI. To study the incorporation of oligonucleotides dT_{18} and dG_{18}, we adapted the method developed by Beavis and Bridson[32] for the study of the incorporation of proteins into crystals of sinapinic acid. In the present experiment, we crystallized sinapinic acid in the presence of acridine orange, which effectively stains single-stranded DNA.[33] Sinapinic acid crystals formed from solutions in which oligonucleotide was absent did not become colored, indicating that acridine orange was not incorporated into the crystals. When dT_{18} was added to sinapinic acid–dye solution, the crystals obtained were intensely orange in color, demonstrating the incorporation of a dT_{18}–acridine orange complex. When dG_{18} was added to the sinapinic acid–dye solution, the crystals obtained were also orange in color with an intensity similar to those obtained in presence of dT_{18}.

The oligonucleotide–dye incorporation experiment indicated that similar amounts of dT_{18} and dG_{18} were included into the sinapinic acid matrix crystals. To obtain a more accurate determination of the relative incorporation of dT_{18} and dG_{18}, we devised a reversed-phase HPLC method using light absorption at 260 nm for quantification. A relatively low recovery (~50%) was observed for dG_{18} from Vydac C_4 and Reliasil C_{18} columns, necessitating the use of one or two post-collection column washes with the eluting gradient. Such low recoveries have been observed previously for oligonucleotides containing $(dG)_n$ motifs and have been suggested to be due to association of bases.[34]

To determine the amount of incorporation into the

matrix, the sinapinic acid crystals grown in presence of acridine orange and either dT_{18} or dG_{18} were dissolved in 20% aqueous acetonitrile and subjected to HPLC. The amount of oligonucleotide incorporated into the matrix crystals from the original 2 μM solution (see Experimental) was determined to be in the range 50–100% for dG_{18} and 75–100% for dT_{18}, demonstrating that the amount of incorporation was the same to within the accuracy of the measurement (a factor of two).

The finding that both dT_{18} and dG_{18} are incorporated into the matrix crystals to comparable extents indicates that lack of incorporation of dG_{18} is not the primary reason for our inability to obtain a mass spectrometric response for dG_{18}. However, this conclusion assumes that the dG_{18}-dye complex is incorporated in the same manner as neat dG_{18} and that the microscopic environment of sample molecules within the crystals and the distribution of oligonucleotide within the crystals are similar for dG_{18} and dT_{18}. The described experiment does not rule out the possibility that differences in the details of incorporation may occur and profoundly influence the mass spectrometric response. Possible causes for microscopic differences in incorporation may arise from a different tendency for association of bases and from differences in the oligonucleotide solution structures.

In aqueous solution, association of bases formed by strong interplanar interactions have been observed, and self-association constants were found to decrease in the order purine–purine > pyrimidine–pyrimidine.[35] Strong self-association of homooligonucleotides may interfere with appropriate incorporation of the oligonucleotide into the matrix.

Differences in solution structures have been observed for oligothymidines and oligoguanosines. For oligothymidines a weak secondary structure with non-stacked bases turned out[36] was observed and may account for appropriate incorporation and therefore successful mass spectrometric analysis of thymidines. Conversely, for oligoguanosines a variety of physical data have suggested a quadruple helical structure.[37]

Fragmentation. Very little mass spectrometric fragmentation is evident in the spectra of oligothymidines in all of the matrices studied. In contrast, the spectra obtained from dG_7 showed evidence for a substantial amount of fragmentation. The degree of fragmentation varied among different matrices and was found to be most pronounced in 2,5-dihydroxybenzoic acid (Fig. 7). A weak molecule-ion was observed at *m/z* 2280. The several peaks at higher mass probably arise from the attachment of adduct species of dG_7. The most intense fragment peaks were found to arise from fragmentation along the sugar-phosphate backbone (primarily at the 3′ C—O bond and the 5′ P—O or C—O bonds). If we assume that the degree of fragmentation increases strongly as a function of polymer length, then fragmentation may, in large part, explain our inability to observe longer stretches of polyguanosine (e.g. dG_{18}).

In order to investigate the role of base structure on the mass spectrometric response, we also analyzed $d(uridine)_{18}$ and $d(inosine)_{18}$. Uridine is related to cytidine by the substitution of the exocyclic 4-amino group by a keto oxygen or to thymidine by the substitution of the 5-methyl group hydrogen. Inosine is related to adenosine by the substitution of the exocyclic 6-amino group by a keto oxygen or to guanosine by substitution of the exocyclic 2-amino group by hydrogen. For dU_{18}, we obtained a mass spectrum very similar to dT_{18} [Fig. 8(a)]. The intense peaks for dU_{18} demonstrate that the 5-methyl group in thymidine does not have a strong influence on the mass spectrometric response. In contrast, the lack of response dC_{18} under identical conditions reveals a major influence of the 4-amino group in

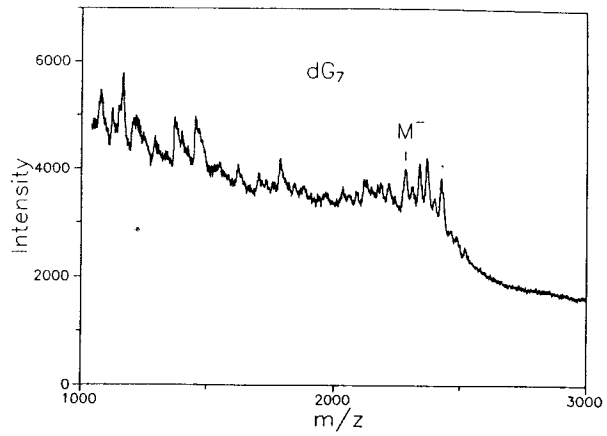

Figure 7. Negative-ion mass spectrum of dG_7 from 2,5-dihydroxybenzoic acid.

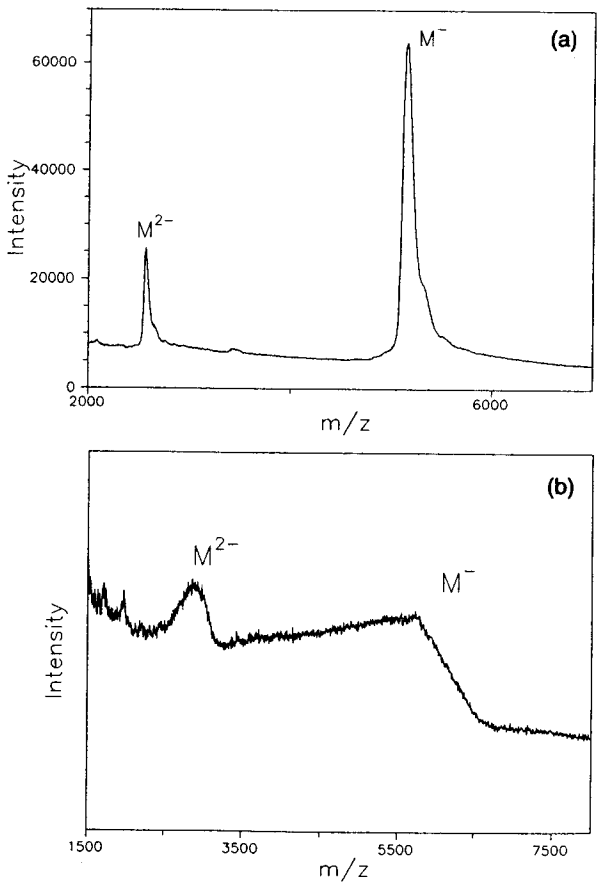

Figure 8. Negative-ion mass spectra of (a) $d(uridine)_{18}$ and (b) $d(inosine)_{18}$ from ferulic acid.

cytosine. The spectrum of dI_{18} exhibited distinct broad peaks at masses approximately consistent with the intact molecule [Fig. 8(b)], demonstrating that substitution of the 2-amino group of guanosine by hydrogen (or substitution of the 6-amino group of adenosine by a keto group) has a dramatic influence on the response (since no trace of molecule-ion peaks are observed for either dG_{18} or dA_{18}). However, the low-mass tailing of the peaks and the decreased width of the doubly charged ion peak compared with the singly charged ion peak demonstrates that a rapid fragmentation of these species occurs during the time required for ion acceleration (0.8–1.2 μs). The presence of ions corresponding to dI_{18} compared to the absence of ions corresponding to dG_{18} (and dA_{18}) may be explained by a lesser tendency for fragmentation of deprotonated dI_{18} as a result of the substitution of the exocyclic amino group. These results for polyuridine and polyinosine indicate a major influence of the exocyclic amino group on the mass spectrometric response of oligonucleotides. Modeling studies are in progress to gain an understanding of interactions between the phosphate diester backbone and the amino group. This may lead to the design of modified nucleotides with lower tendency for fragmentation. Results obtained for methylphosphonate oligonucleotides indicate a much lower influence of base composition[38] and might be due to a lesser tendency of the methylphosphonate oligonucleotides for fragmentation.

CONCLUSION

We have reported a survey of potential matrix materials for their usefulness in the laser desorption mass spectrometric analysis of oligonucleotides. Of 48 compounds investigated, 20 yielded a discernible signal of intact dT_{18}. None of these matrices yielded a discernible signal of dG_{18}, demonstrating that the previous observation of the influence of base composition on the mass spectrometric response[13] is consistently observed for a wide variety of structurally different matrices. We investigated three possible causes for the lack of mass spectrometric response for polyguanosines in contrast to the strong response for polythymidines, and inferred that fragmentation was a likely main contributor. Although we observed an overall incorporation into matrix crystals for dG_{18} comparable to that of dT_{18}, inappropriate incorporation on the microscopic scale of dG_{18} (possibly caused by base association or details in solution structures) may also inhibit its non-destructive desorption and ionization.

The analysis of the modified oligonucleotides $d(uridine)_{18}$ and $d(inosine)_{18}$ indicated an influence of the exocyclic amino group on the mass spectrometric response. Further studies of other modifications of dG, dA and dC may lead to a deeper understanding of the influence of particular structural elements on the mass spectrometric response. The projected use of MALDI for DNA sequencing requires considerable further improvements to the mass spectrometric response of MALDI for DNA fragments. Such improvements may be achievable by appropriate modification of the nucleotides (either before or after Sanger chain extension/chain termination) or by discovery of improved matrix materials and sample preparation methods.

Note Added in Proof:
Significant improvements and findings have been made since submission of the manuscript, which we feel should not go unmentioned. The use of ammonium citrate to suppress peak broadening due to multiple alkali ion adducts [U. Pieles, W. Zürcher, M. Schär and H. E. Moser, *Nucleic Acid Research* **21**, 3191 (1993)] has greatly improved the quality of mass spectra of oligonucleotides. For example, our analysis of dT_{18} in presence of ammonium citrate yielded only one well resolved singly-charged peak with a mass of 5414 u, thus indicating that the observed mass increase of 126 u (Fig. 3) was likely caused by multiple alkali ion adduction. G. R. Parr, L. Zhu, M. C. Fitzgerald and L. M. Smith (*Proceedings of 41st ASMS Conference on Mass Spectrometry*, San Francisco, 1993, p. 788) have demonstrated with great clarity that fragmentation of the phosphodiester backbone of oligodeoxyribonucleotides containing multiple deoxythymidines and deoxyguanosines occurs preferentially adjacent to the deoxyguanosines. E. Nordhoff, R. Cramer, M. Karas and F. Hillenkamp, F. Kirpekar, K. Kristiansen, and P. Roepstorff, (*Nucleic Acids Research*, **21**, 3347 (1993)), have recently considered the ion stability of nucleic acids in infrared matrix-assisted laser desorption/ionization mass spectrometry.

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