A procedure for selective DNA alkylation and detection by mass spectrometry

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ABSTRACT

A method which improves the detectability of DNA by mass spectrometry is presented. By quantitatively alkylation of the backbone of phosphorothioate oligonucleotides, the problems of gas phase ion generation by matrix assisted laser desorption ionization can be controlled. We have developed a selective alkylating protocol for phosphorothioate oligonucleotides which is a facile way of generating non-ionic nucleic acids. A variety of alkylating agents were studied and their kinetics were monitored in a gel electrophoretic assay and by mass spectrometry.

INTRODUCTION

The introduction of matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI MS) (1) has opened new possibilities for the application of mass spectrometric analysis in molecular biology and, increasingly, in DNA analysis (2). DNA has proven to be significantly more difficult to analyze by MALDI MS than peptides and proteins (3) due to very different mass ranges and the quantitative number of negative charges that have to be controlled for mass spectrometric analysis. The displacement of sodium by ammonium counter ions or very stringent desalting protocols improve the resolution significantly (4,5). Using this improvement, restriction enzyme digested fragments can be analysed by matrix assisted laser desorption (6). Chemically synthesized methylphosphonate oligonucleotides which have an uncharged backbone show excellent definition by MALDI MS (7,8). They were detected in positive ion mode while for the analysis of negatively charged oligonucleotides negative ion mode detection was preferable.

The partial alkylation of phosphorothioate containing oligonucleotides for selective chemical backbone cleavage has been described (9,10) and fluorescent reporter groups have been linked to oligonucleotides via phosphorothioate groups (11,12). Extensive work on the enzymatic incorporation of α-thio nucleotide triphosphates by DNA polymerases has been done for the development of alternative sequencing protocols (13,14).

Here, we show the chemical post-synthesis modification of phosphorothioate oligonucleotides in order to displace negative charges from the sugar phosphate backbone prior to mass spectrometric analysis. Quantitative alkylation of an oligonucleotide reduces the number of gas phase events required and allows the analysis by MALDI in positive ion mode. Phosphorothioate linked oligonucleotides were chosen because quantitative chemical modification of the regular sugar phosphate backbone of DNA is not feasible while on the other hand phosphorothioate DNA can be generated enzymatically.

MATERIALS AND METHODS

The phosphorothioate deoxyoligonucleotides listed below were synthesized by an in-house oligonucleotide synthesis service by standard phosphoramidite chemistry: HO-CCC, HO-TTT, HO-AAA, HO-GGG, HO-AGCT, HO-TTTTTTTTTT, HO-TCGATCGATCGA, M13mp18 forward sequencing primer HO-GTAAAACGACGGCCAGT. Regular HO-TTTTTTTTTT was also synthesized.

Phosphorothioate 5'-hexyl-dAGCT was custom synthesized by Oswel DNA Service, University of Edinburgh (Edinburgh EH9 3JJ, UK) using standard phosphoramidite chemistry. A hexyl building block (C₆H₁₃) was coupled to the 5' end also via a PT link.

Deoxyadenosine 5'-α-thiotriphosphate (Sp-isomer, dATP-α-S), deoxycytidine 5'-α-thiotriphosphate (Sp-isomer, dCTP-α-S), deoxyguanosine 5'-α-thiotriphosphate (Sp-isomer, dGTP-α-S) thymidine 5'-α-thiotriphosphate (Sp-isomer, dTTP-α-S) were purchased from Amersham International (Little Chalfont, UK). CH₃I, C₂H₅I, C₃H₇I, 2,3,4-trihydroxyacetophenone, 2,4,6-trihydroxyacetophenone, sinapinic acid and L-tartaric acid diammonium salt were purchased from Aldrich (Gillingham, UK).

5' End-labelling (³²P) of PT oligonucleotides was done with T4 polynucleotide kinase according to the protocol of Mundi et al. (15). T4 polynucleotide kinase was purchased from New England Biolabs (Bishop’s Stortford, UK) and [γ-³²P]dATP from Amersham International (Little Chalfont, UK).

Alkylation of phosphorothioate linked oligonucleotides

The thio specific alkylation of PT oligonucleotides (Fig. 1) was evaluated in different solvent systems (data not shown) with a series of alkylating agents (CH₃I, C₂H₅I, C₃H₇I). Efficient alkylation was achieved in 10% v/v 30 mM Tris—HCl pH 8.0 + 90% dimethylformamide, final pH 8.6 (Tris—DMF). Reactions were done in closed 0.5 ml Eppendorf tubes. 5 µl of Tris—DMF,

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1 µl of the PT oligonucleotide (~1 mM, 1 nmol) and 1 µl of the alkylating agent (~10 nmol) were mixed and placed in a water bath at 55°C. 1 µl samples were taken at different time intervals and analyzed by MALDI MS or gel electrophoresis. The reaction time for efficient alkylation was CH₃I < C₂H₅I < C₃H₇I. CH₃I was more difficult to handle than the other alkylating agents due to its low boiling point of 43°C and some of it might have been lost during sampling, although sampling was done at room temperature. With C₂H₅I the addition of alkylating agent (0.5-1 µl) after each sampling led to full alkylation of the PT backbone in less than 1 h at 55°C. The reaction time could still be further reduced by raising the reaction temperature. A control reaction under the same conditions (pH, temperature) but without DMF did not result in any alkylation of the PT groups.

Gel assay
Oligonucleotides were separated on 20 cm x 0.4 mm 15% polyacrylamide gels containing 8 M urea. They were run for 1–2 h at 40 W, fixed for 20 min in 10% acetic acid, dried and X-ray film (Fuji RX) exposed for 5 h.

Matrix Assisted Laser Desorption Ionization time-of-flight mass spectrometry (MALDI MS)
Equivalent amounts of crude reaction mixture and 2% solutions of sinapinic acid (SA) in 50% aqueous acetonitrile (usually 1 µl each) were mixed. 0.5 µl (containing ~35 pmol of product) was spotted onto the target slide and air dried. Solvents and volatile alkylating agents (CH₃I, C₂H₅I, C₃H₇I) evaporated upon formation of the matrix while the product was embedded in the matrix. Starting materials and products were also analyzed using a 2% 2,3,4-trihydroxyacetophenone (234-THA) or 2,4,6-trihydroxyacetophenone (246-THA) matrix in 50% aqueous ethanol with 100 mM l-tartaric acid diammonium salt added according to the method of Pieles et al. (5). Mass spectra were recorded on a Finnigan MAT LaserMat 2000 time-of-flight mass spectrometer, essentially described by Mock et al. (16). Although the mass spectrometer was not internally calibrated the resolution was usually better than ±2 Da in the mass range below 4000 Da which was sufficient for these experiments.

RESULTS
The alkylation of phosphorothioate oligonucleotides
Phosphorothioate oligonucleotides were alkylated very efficiently using a 90% DMF solution containing 3 mM Tris–HCl pH 8.0, final pH 8.6 at 55°C (Fig. 1). C₂H₅I was found to be a very
appropriate alkylation agent for this purpose. Under these conditions the reaction of C$_2$H$_5$I with PT oligonucleotides was complete within 1 h. The alkylation rate seems to be independent of the length of the oligonucleotide (Figs 2 and 3).

**Analysis of alkylated phosphorothioate oligonucleotides by gel electrophoresis**

In addition to MALDI MS analysis (described below), the alkylation products were studied by gel electrophoresis using $^{32}$P-end labelled PT oligonucleotides. Removal of charges from the backbone which corresponds to alkylation reactions were monitored by reduced mobility of the oligonucleotide in gel electrophoresis. Individual alkylation steps can be seen as a ladder of oligonucleotides with decreasing number of charges. Figure 2 shows the time course of the alkylation of a $^{32}$P-end labelled PT 17mer (M13mp18 forward sequencing primer) with C$_2$H$_5$I in Tris–DMF. The effect of adding C$_2$H$_5$I after each time the reaction is sampled is clearly visible. Fully alkylated material can not migrate into the gel due to its lack of charge and therefore, will largely be lost during the work up of the gel (fixed in 10% acetic acid). The inefficiency of alkylation of the phosphate label (estimated to be ~40%) results in two residual bands on the gel. However, alkylation products that are not associated with the alkylation of the PT groups, and therefore do not correspond to the removal of charges, could not be detected in this assay but by MALDI MS. A control alkylation of the same PT 17mer at pH 8.6 in protic solvent did not lead to the formation of backbone alkylation products.

**Comparison of MALDI MS detection in positive and negative ion mode**

The kinetics of alkylation of PT oligonucleotides were followed by MALDI MS both in positive ion mode (PIM) and negative ion mode (NIM). During the reaction the different PT alkylation steps could be observed in NIM with exception of the fully alkylated PT oligonucleotide. Upon completion of the alkylation of the PT groups no signals at all could be detected in NIM. The fully alkylated PT oligonucleotide could be analyzed in PIM with improvingly excellent resolution during the reaction (Fig. 4).

The selectivity of the alkylation of PT oligonucleotides was studied on the set of 3mers, HO-TTT, HO-CCC, HO-GGG, HO-AAA and HO-TCGA (all PT linked) were alkylated in Tris–DMF and analyzed by MALDI MS using sinapinic acid, 2,3,4-trihydroxyacetophenone and 2,4,6-trihydroxyacetophenone as matrices (Table 1). Spectra were recorded in PIM and NIM as described above. Alkylation with CH$_3$I was slow in comparison. Only products of the mass of the phosphorothioate oligonucleotide with no more than the number of phosphorothioate groups +1 times ethylated were observed. This indicates that one or both of the end hydroxy groups were alkylated in addition to all phosphorothioate groups. Alkylation with C$_3$I was slow in comparison. Only products of the mass of the phosphorothioate oligonucleotide with no more than the number of phosphorothioate groups +1 times alkylated were observed.

**Table 1. Masses of alkylated phosphorothioate oligonucleotides**

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Measured and calculated masses of phosphorothioate oligonucleotides and their alkylation products formed in 3 mM Tris–HCl in 90% DMF at pH 8.6. MALDI MS analysis of the native phosphorothioate oligonucleotides, that gave the masses of the free acids, were carried out in 2,3,4-trihydroxyacetophenone matrix after addition of L-tartaric acid diammonium salt. The alkylation products were analyzed using sinapinic acid as matrix. [-] indicates that no signal could be detected, while empty positions were not attempted: With CH$_3$I multiple higher methylation was found with purine bases and assigned to the alkylation of bases. With C$_2$H$_5$I negative ion mode analysis showed a product during the reactions with the mass of the free acid of the phosphorothioate oligonucleotide plus the number of PT groups —1 times ethylated. The endpoint of alkylation was determined by the disappearance of negative ion mode signal. In positive ion mode products with a mass of the free acid of the oligonucleotide plus the number of phosphorothioate groups +1 or +2 times ethylated were observed. This indicates that one or both of the end hydroxy groups were alkylated in addition to all phosphorothioate groups. Alkylation with C$_3$I was slow in comparison. Only products of the mass of the phosphorothioate oligonucleotide with no more than the number of phosphorothioate groups +1 times alkylated were observed.
the alkylation of adenine and guanine while cytosine and thymine remained unreacted. For CH₃I no solvent conditions were found under which the alkylation was selective for the PT groups. Propylation with C₃H₇I was found to be comparatively slow. It is conceivable that a protocol with higher temperatures could be worked out as the propylation was selective for PT and no base alkylation was observed. C₂H₅I was very practical under these conditions. It did not alkylate any of the four bases, but efficiently ethylated the phosphorothioate backbone. The higher boiling point of C₂H₅I than CH₃I made its handling significantly easier. The addition of alkylating agent after sampling helped drive the backbone alkylation to completion without leading to base alkylation. Due to the small reaction volumes it is probable that some of the alkylating agent is lost during sampling. It was found that the 5'- and 3'-hydroxy groups were susceptible to alkylation by CH₃I, C₂H₅I and to a lesser extent by C₃H₇I although these alkylations were not quantitative.

In order to prevent alkylation of the 5'-end, oligonucleotides were designed where the 5'-OH is replaced by a 5'-hexyl chain linked to the oligonucleotide via a PT group. Figure 3 shows the time course of the ethylation of PT 5'-hexyl-AGCT, monitored in PIM and NIM in three different matrices (SA, 234-THA and 246-THA). In all three matrices the appearance of signals at 1514 Da and 1542 Da (four and five times alkylated starting material) was detected in PIM. In NIM a signal at 1484 Da (three times alkylated starting material) was detected in SA and 246-THA matrix. This signal disappeared in the course of the reaction. Using 234-THA and NIM no signal could be detected during the reaction. Figure 4 shows the PIM and NIM spectra after completion of the alkylation using SA. In this experiment the 3'-OH was found to be completely alkylated although often a double peak with masses corresponding to four and five times alkylated starting material was observed in PIM indicating that the 3'-OH alkylation is not quantitative. However it was considered obsolete to constrain the alkylation of the 3'-OH as in the Sanger sequencing chemistry the terminating dideoxy bases lack this group anyway.

Figure 4. MALDI mass spectra of the alkylation product of phosphorothioate 5'-hexyl-AGCT with G₂H₅I in 3 mM Tris-HCl in 90% dimethylformamide after 3 h at 55°C recorded in (A) positive ion mode and (B) negative ion mode. Sinapinic acid was used as matrix. Full alkylation of the 3'-OH groups was achieved in the shown spectra. Often incomplete alkylation of 3'-OH is observed and manifests itself as a double peak, the two peaks differing by the mass of the alkyl group.

Ethylation products of longer PT oligonucleotides could be detected by MALDI MS. HO-T₁₀ (PT linked) gave a well resolved signal that corresponded to eleven alkylations in PIM using SA as matrix. No corresponding signal was detected in NIM after completion of the alkylation which indicates optimal conditions for alkylation. The control alkylation of a regular HO-T₁₀ in Tris-DMF gave a product that corresponded in mass to the four times ethylated starting material this indicates ~40% efficiency of alkylation of regular phosphate groups under these conditions. It could only be detected in NIM. PT HO-(TCGA)₃ could be ethylated to completion under the same conditions. The signal detected in PIM was very symmetrical, well resolved and corresponded to the starting oligonucleotide ethylated 12 times. During the reaction the decreasing signal of the 10 times ethylated intermediate could be detected in NIM. Products with fewer than 10 alkylations could never be resolved during the alkylation indicating that they are either never abundant enough for detection or that higher alkylation products are more easily detected by MALDI MS due to their homogeneity.

Throughout, signals of complete alkylation products detected in PIM were stronger than the signals of starting materials. The ratio strongly depended on the matrix.

Salts, like NaI, which is a side product of the alkylation reaction, and buffers, were not removed. This might lead to a certain degree of suppression for larger oligonucleotides but could not be observed with the instrument used.

A mixture of ethylated HO-T₃₅, HO-AGCT and HO-T₁₀ (all PT linked) was analysed by MALDI MS using sinapinic acid as a matrix (Fig. 5). Excellent separation and definition of signals was observed. The instrument specific resolution number slightly decreased to m/Δm = 120 for the 10mer. Interestingly the analysis of a mixture of starting material with an equal concentration ratio of the three oligonucleotides, analyzed in 246-THA (with the addition of 100 mM L-tartaric acid diammonium salt) in NIM, yielded a different intensity ratio. The alkylated mixture gave HO-T₃₅ > HO-T₁₀ > HO-AGCT while the starting material gave...
Figure 5. MALDI mass spectrum of a mixture of ethylated phosphorothioate HO-TTT, HO-AGCT and HO-T10. Alkylation was done with C2H5I in 3 mM Tris–HCl 90% dimethylformamide at 55°C for 2 h. Sinapinic acid was used as matrix and the spectrum recorded in positive ion mode. Masses detected are 967, 1336 and 3408. The resolution number m/Δm is 160 for the lower peaks and 120 for the 10mer.

HO-AGCT > HO-TTT >> HO-T10. Clearly there is a significant difference in the mode of ionization which might account for this observation.

DISCUSSION

Using 3mers simplifies the MS analysis of alkylation products as there are only three distinguishable alkylation positions with different multiplicities and detectability in alternate ion modes (two bridge phosphorothioate groups, two end OH groups and three bases). Base and end alkylation can be analyzed in negative ion mode while complete backbone alkylation products can be detected in positive ion mode. The alkylation of the end groups was studied using end modified mixed 4mers (PT 5'-hexyl-AGCT). We found that signal definition could be improved by this substitution. Alkylation at different positions leads to an overall higher number of alkylations and detection of larger products. For example (Table 1) in the exhaustive alkylation of PT HO-AAA with CH3I the addition of six alkyl groups was detected in PIM (two bridge PT groups, two or three bases and one or two end OH groups). This indicates that the methylation of the PT groups runs to completion but bases are also methylated under these conditions.

For C2H5I the highest number of alkyl groups added to the PT trimer was four. The reaction could not be driven any further. Usually mixtures of three and four times ethylated trimers were found in positive ion mode without a corresponding negative ion mode signal. While no base ethylation takes place the reaction with bases is not complete, but bases are also methylated under these conditions.

One of the main problems of MALDI MS of oligonucleotides is the decrease in resolution for increasing length of the oligonucleotide (17,18). Both in this study and before (7,8) it has been found that non-ionic oligonucleotides give good definition of signals even in mixtures. We have tried to visualize the process of MALDI of oligonucleotides and have developed the following model for plausibility (Fig. 6):

(i) Desorption: the laser induced desorption process produces a situation where hot and charged species originating from the matrix material diverge interspersed with cold overall charge neutral oligonucleotides. Charge separation starts taking place due to the electric field that the molecules in the plume are exposed to. The oligonucleotides stay associated with their counter ions.

(ii) Ion exchange: collisions of hot matrix material with the analyte lead to the displacement of Na+ from the sugar–phosphate backbone. The negative charge of the phosphate is saturated by molecules with high gas phase acidity in a concerted event. The possibility for the dissipation of energy of matrix fragments decreases as the particle density decreases.

(iii) Ionization: the ionization process that gives a charge to the oligonucleotide, required for the acceleration, takes place when the concentration of strong gas phase acids drops below a critical concentration and the probability for a concerted event gets small. In a sense ionization could be viewed as a failure of the ion exchange process.

(iv) Acceleration: the charged oligonucleotide is accelerated towards the detector.

With increasing length of an oligonucleotide the number of ion exchange events increases. Incomplete exchange results in broadening of the signal. Often Na+ adducts have been observed although usually the predominant peak detected corresponded to the mass of the free acid. The amount of matrix material consumed increases linearly with the concentration of bases and might therefore reach a level of saturation where no further ion exchanges can take place. This might explain why mixtures of oligonucleotides show poorer signals for the larger oligonucleotides while the same large oligonucleotides analyzed on their own show better resolution (17). The resolution/definition is a function of the overall concentration of analyte and therefore a function of the number of ionic positions in an oligonucleotide. In a mixture of oligonucleotides a large oligonucleotide requiring more collisions will feel the effect of an exhausting matrix most strongly. A logical, but not yet feasible solution, would be to lower the concentration of oligonucleotide analyte material but, of course, this decreases the detectability. With analyte to matrix ratios of 1:100 (19) very efficient exchange would have to be achieved in order to obtain reasonable signal with longer oligonucleotides (>20 bases). Higher ratios in favour of the matrix (1:104) have been used and this definitely helps to improve the probability of a successful quantitative exchange (20). The time frame in which the ion exchange takes place is of significance for the resolution. If the ion exchange is rapid and the generation of an ion thereafter efficient, one can expect good resolution. With time passing after the vaporization process the probability of collisions decreases exponentially. It has been found that the addition of NH4+ significantly increases the definition of a signal (13). This is in accordance with our model in that NH4+ ions upon displacement from the sugar–phosphate backbone, themselves act as acids due to their high gas phase acidity and provide the protons back to the oligonucleotide. If ionization processes were predominant over ion exchange processes one would expect to see larger amounts of doubly and triply charged species. Usually predominantly singly charged species are observed. On the other hand if exchange events were more highly efficient than ionization events within the model described one would obtain the charge neutral free acid of an oligonucleotide and that would most likely be detected in positive ion mode after base protonation as the gas phase acidity of the matrix has to be high for rapid exchanges.

The gas phase interaction of molecules and fragment molecules is of vital importance to the analysis of oligonucleotides by MALDI. By selective alkylation which leads to complete removal of negative charges from the PT oligonucleotide backbone, we manage to reduce the number of gas phase events between vaporization and detection. The advantage of low numbers of
Figure 6. Model for the gas phase behaviour of oligonucleotides during matrix assisted laser desorption ionization. (A) After desorption of the matrix a plume containing a mixture charged, neutral, hot and cold molecules start interacting with each other. (B) Collisions with the native oligonucleotide lead to the release of sodium cations. The negative charges on the oligonucleotides are immediately neutralized by collisions with compounds with high gas phase acidity. (C) The plume reduces due to molecules that have been accelerated out of it. Collisions with the oligonucleotides still lead to the release of sodium but saturation of the residual negative charge on the sugar-phosphate backbone of the oligonucleotide no longer takes place. (D) The oligonucleotide with the negative charge is accelerated out of the plume.

Recentley Nordhoff et al. have found that N-glycosidic bonds are more prone to break in DNA than RNA during MALDI resulting in the loss of a base (4). They propose that the 2'-OH group in RNA stabilizes the N-glycosidic bond while in DNA the phosphate proton is transferred to the base which leads to the breakage of the N-glycosidic bond. Our alkylation protocol leads to a completely new arrangement of groups on an oligonucleotide backbone and a significant decrease in the acidity of the backbone. No breakage of the alkylated backbone was observed in our MALDI MS analysis.

We are currently completing the enzymatic generation of phosphorothioate oligonucleotides and intend to implement this protocol for typing and sequencing DNA. We are also evaluating the use of alkylated phosphorothioate oligonucleotides as antisense DNA. The simple and effective procedure for removing charges from the oligonucleotides is very enticing for this purpose.

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REFERENCES