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Negative electrospray ionization mass spectrometry of synthetic and chemically modified oligonucleotides

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ABSTRACT

We report here on the analysis of synthetic oligonucleotides by electrospray ionization mass spectrometry (ESI-MS). After intensive removal of salt ions (especially sodium cations), negative ion mass spectra, allowing mass measurement with an accuracy of 0.01%, were obtained on several oligonucleotides up to 80 nucleotides. In most cases, the resolution was sufficient to observe n-1 and n-2 forms due to internal deletions during automated synthesis, and to identify the missing nucleotides. A 132-mer, whose size is close to the limit of automated chemical synthesis, was also successfully mass measured. A quantitative study showed that ESI-MS can provide quantitative data on oligonucleotides of similar size and structure. The described methodology is used to characterize oligonucleotide analogues such as phosphorothioate oligonucleotides designed for antisense applications. Finally, analyses in the positive ion mode on a trimer TpTpT in the presence of different amine bases were performed and allowed a better understanding of the influence of these bases on the ions formation.

INTRODUCTION

Over the last 60 years, the study of nucleic acids has become increasingly important because of their central role in many biological processes. With the rapid development of molecular biology and its impact on all areas of life sciences as well as with the development of the biotechnology industry, powerful analytical methods are needed to determine the structure and purity of nucleic acids and their constituents. In particular, chemically synthesized oligonucleotides have found widespread use in molecular biology as for example primers in DNA sequencing and DNA amplification by the polymerase chain reaction as well as in site-directed mutagenesis. More recently, synthetic oligonucleotides have been conceived as potential therapeutic agents and some of them are undergoing clinical evaluation (1,2).

With the development of these new applications, an accurate and detailed knowledge of the structure of the employed oligonucleotides is required and it has become more and more important to find tools which are able to characterize both natural and synthetic oligonucleotides at the structural level. This means confirmation of the sequence of synthetic oligonucleotides, detection and quantification of structurally related impurities as well as full structural characterization of these impurities. A somewhat paradoxical situation has developed in the field of chemically synthesized oligonucleotides, where hundreds of different molecules can be automatically synthesized on a single instrument in the time of a few months while complete analytical characterization of even a single oligonucleotide may take weeks. In addition, commonly employed methods used to analyze synthetic oligonucleotides can be very laborious and may often require the use of radioactivity (e.g. Maxam-Gilbert sequencing; 3). In this context, rapid and reliable analytical methods are needed especially with respect to the characterization of chemically modified oligonucleotides (e.g. conjugates of oligonucleotides with other classes of compounds such as peptides, metal chelators, etc.). Accurate molecular mass measurements would help to achieve this goal by allowing to confirm that the observed mass corresponds to the expected structure. As for protein studies, mass spectrometry could become a quick and reliable method for DNA analysis, synthetic modification studies and diagnostic applications.

The applications of mass spectrometry to the analysis of nucleic acids have long been limited by experimental problems relating to their very high polarity and polyanionic nature. The first studies using fast atom bombardment (FAB) or liquid secondary ion mass spectrometry (LSIMS) were limited to small oligonucleotides (4; <5000 Da). With the development of new methods of ionization such as electrospray ionization (ESI; 5) or matrix-assisted laser desorption ionization (MALDI; 6–8), which can produce intact gaseous ions from highly polar molecules, it has become possible to analyze higher molecular weight oligonuleotides ($>10\,000$ Da) with a precision comparable with that obtained for proteins (9,10).

However, up to the present day, it is quite difficult to perform mass measurements on oligonucleotides by either ESI or MALDI, especially if the molecular weight surpasses 10 kDa. The main difficulties arise due to the formation of oligonucleotide-sodium or -potassium adducts which persist after ionization and desolvation (11,12). The presence of sodium adducts becomes

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a major problem in the case of large oligonucleotides where the resolution of the generally employed quadrupole mass analyzers is insufficient to resolve the different sodium adduct peaks leading to erroneous average molecular mass measurements. Reduction of the amount of sodium adducts is thus critical to obtain high quality spectra. Replacement of sodium ions with ammonium has been shown to considerably reduce sodium adduct formation in ESI-MS (5). Extending this work, we show here that high quality mass spectra up to 25 kDa (80-mer oligonucleotides) can be obtained on a routine basis and that an upper limit of 132 nucleotides of a chemically synthesized oligonucleotide was reached. Comparing ESI-MS and capillary gel electrophoresis showed that ESI-MS can provide quantitative data on related contaminants of the major product. Furthermore, different chemically modified oligonucleotides have been successfully analyzed. Analyses of a trinucleotide diphosphate in the presence of different amines under positive ionization conditions allowed to establish an order of stability of the respective adducts, and to gain some insight into the effects of amines on the persistence of sodium-oligonucleotide adducts.

MATERIALS AND METHODS

Chemicals

Ammonia, trimethylamine (TMA) and triethylamine (TEA) were purchased from Pierce (Oud Beijerland, the Netherlands). Horse heart myoglobin and thymidylyl (3′-5′)-thymidylyl-(3′-5′) thymidine (TpTpT) were obtained from Sigma (St Quentin Fallavier, France). The FAM amidite was obtained from Perkin-Elmer (Roissy, France). Oligodeoxynucleotides were synthesized on a 0.2 μmol scale using solid-phase phosphoramidite chemistry (13,14) on either a model 380B (Applied Biosystems, Foster City, CA, USA) or a model 7500 (Milligen, Bedford, MA, USA) automated synthesizer. Oligonucleotides were purified by reversed-phase HPLC before removing their 5′-dimethoxytrityl protecting groups (C_{18} μBondapak, 10 μm particle size, 12.5 nm pore size) using a gradient of 20−30% (<50 nucleotides) or 5−30% (>50 nucleotides) acetonitrile in 100 mM triethylammonium acetate, pH 6.9. The 6-mer and 18-mer phosphorothioate oligonucleotides were purified by reversed-phase HPLC after removing the protecting groups using a gradient of 9−15% acetonitrile in 10 mM triethylammonium acetate, pH 5.5.

Electrospray ionization mass spectrometry

ESI-MS spectra were obtained on a Bio-Q triple quadrupole mass spectrometer (Fisons, Manchester, UK) with a mass of range of 4000 Thomson. In most cases, samples were dissolved in aqueous 50% acetonitrile (v/v) containing 1% triethylamine at a final concentration of 20−50 pmol/μl. 10 μl aliquots were introduced into the ion source at a flow rate of 5 μl/min. The extraction cone voltage was usually set to 50 V and the source temperature to 70°C. Data were acquired in the positive or negative ionization mode from m/z = 500 to m/z = 1500 in 10 s. Calibration was performed in the positive ionization mode using the multiply charged ions produced by a separate introduction of horse heart myoglobin and the resolution adjusted so that the peak at m/z = 998 was 1.5 Da wide on the base. Electrospray parameters in the negative ionization mode were adjusted by injecting a solution of TpTpT in aqueous 50% acetonitrile at a final concentration of 20 pmol/μl.

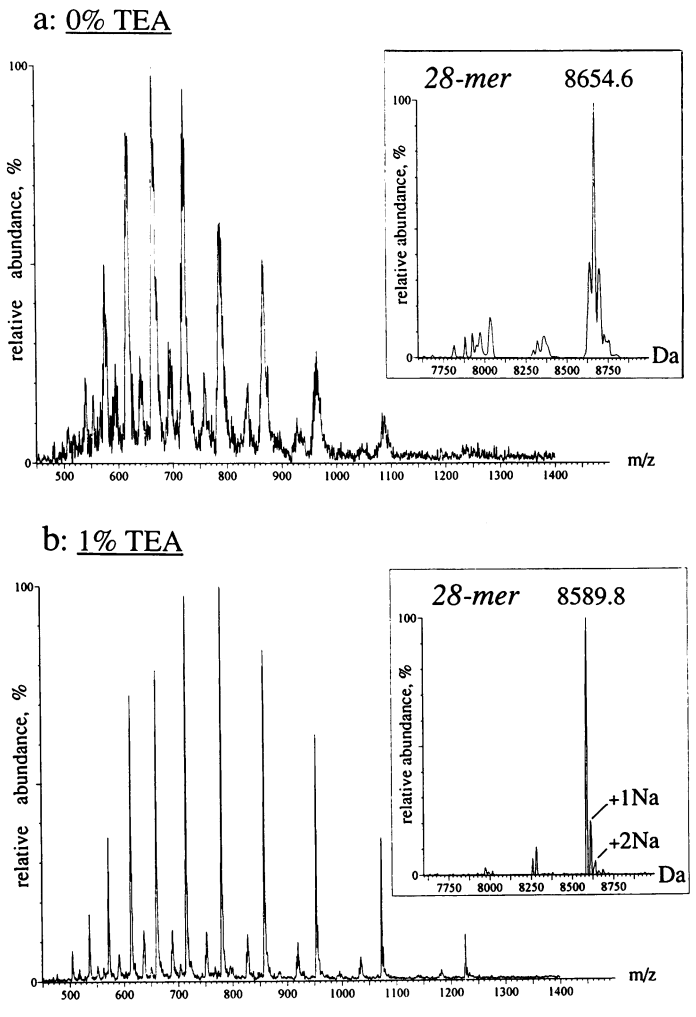

Figure 1. Negative ESI-MS of a synthetic 28-mer oligonucleotide (3′ATCGTTA-CGGCATTAGCAGCTTGAGCAC5′; expected mass: 8588.9 Da) with and without TEA (TEA). The oligomer was dissolved in either H_2O/CH_3CN (**a**) or in H_2O/CH_3CN, 1%TEA (**b**). Real mass scale spectra (obtained after treatment with the Maximum Entropy program) are also presented. The peak width and the mass precision show the improvement due to the addition of 1% TEA.

The Maximum Entropy program which is part of the MassLynx software (Version 1.6.1, VG Fisons, Manchester, UK) was used to reconstitute a real mass scale spectrum from a multiply charged ion mass spectrum obtained by ESI-MS. This special data treatment may allow to separate overlapping peaks depending on the signal-to-noise ratio, the peak width, the separation between the peaks and their intensity ratio.

Sample preparation

Purified oligonucleotides were deprotected with 20% acetic acid and ether extracted. The aqueous phase was lyophilized and the oligonucleotides (1 absorbance unit (OD) per ml at 260 nm) were redissolved in 50 μl Milli Q water, sonicated and treated with 50 μl 10 M ammonium acetate for 2 h at room temperature. Oligonucleotides were precipitated overnight at −20°C by adding 3 volumes of cold ethanol. This procedure was repeated at least three times in order to enhance the displacement of sodium ions. The precipitated oligomers were centrifuged, washed in 80% cold

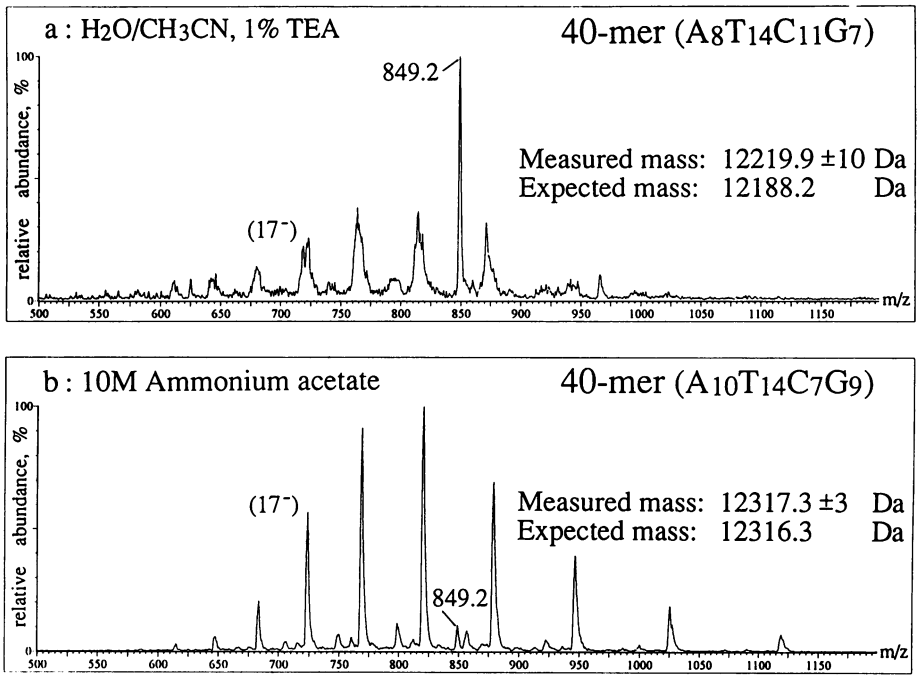

Figure 2. Multiply charged ion mass spectrum of a 40-mer ($A_8T_{14}C_{11}G_7$) dissolved in an aqueous 50% acetonitrile solution containing 1% TEA (**a**). Multiply charged ion mass spectrum of a 40-mer ($A_{10}T_{14}C_7G_9$) after treatment with 10 M ammonium acetate and precipitation with 80% cold ethanol (**b**). An efficient removal of sodium was achieved leading to an accurate mass measurement. The signal at 849.2 corresponds to the internal standard TpTpT.

ethanol and reconstituted to a final concentration of 20–50 pmol/μl with aqueous 50% acetonitrile containing 1% TEA.

Relative quantification of oligonucleotides by ESI-MS and capillary gel electrophoresis

ESI-MS. After synthesis and purification both oligonucleotides (27- and 28-mer) were treated with 10 M ammonium acetate as described above and redissolved in aqueous 50% acetonitrile containing 1% triethylamine. The final concentration of the 28-mer was kept constant at 20 pmol/μl and the concentration of the 27-mer was adjusted to obtain concentration ratios of 0, 0.125, 0.230, 0.330, 0.410 and 0.670 relative to the 28-mer. Mass intensity ratios were calculated for the same charge state $[M-12H]^{12-}$ in the resulting multiply charged ion mass spectra.

Capillary gel electrophoresis. Separations were performed on a P/ACE 2100 system (Beckman Instruments, Palo Alto, CA, USA) working in reversed-polarity (cathode at the capillary inlet). Separations were monitored at 254 nm. Capillaries were thermostated using the liquid cooling system provided with the instrument. Data acquisition was done on a 386/33 microcomputer using System Gold software (Beckman Instruments). Gel-filled capillaries (100 μm internal diameter) were obtained from J&W Scientific (Folsom, CA, USA; 3% T, 3% C polyacrylamide) and prerun for 45 min by slowly increasing the voltage to 250 V/cm (~6 μA).

RESULTS

Electrospray mass spectrometry of synthesized oligonucleotides and general 'desalting procedure'

The first analyses of oligonucleotides by ESI-MS were performed in 1988 by Covey (15) who observed the formation of negative multiply charged ions for a 14-mer (molecular weight ~4.5 kDa). The extension of these measurements to higher molecular weights has been attempted by a number of laboratories and it was pointed out that contamination of the samples with salts (mainly sodium) was a problem of considerable importance. Because of their highly charged nature, oligonucleotides have a tendency to bind sodium ions very tightly even if present in trace amounts. This problem becomes more important for larger oligonucleotides where the different adduct peaks cannot be resolved one from each other, which results in wide peaks corresponding to molecular weights exceeding the mass of the oligonucleotide. More than for proteins, an efficient method for salt removal is thus required.

Electrospray mass spectrometry of synthetic oligonucleotides of less than 10 kDa. The strategy used for reducing the amount of sodium adducts involves the replacement of sodium ions by ammonium ions which appear to be less tightly bound to the nucleotide when ionized in the gas phase. Partial displacement of sodium was indeed observed when adding ammonia to the sample solution resulting in improved mass spectra. Comparing different bases (ammonia, TMA, TEA and diisopropylethylamine) at a concentration of 1% (v/v) showed that TEA appeared to be most efficient. Figure 1 shows the improvement of the obtained mass spectrum for a 28-mer oligonucleotide after dissolving in H_2O/CH_3CN (50/50), 1% TEA as compared with H_2O/CH_3CN (50/50). While the amount of complexed alkali metal ions greatly decreased, complete removal was not achieved. After deconvolution with the Maximum Entropy program, two sodium adducts were still detectable (Figure 1b). It thus appears that the ion pairs formed between the phosphate groups of the oligonucleotide and the triethylammonium ions have been destroyed during the ionization/volatilization process since no

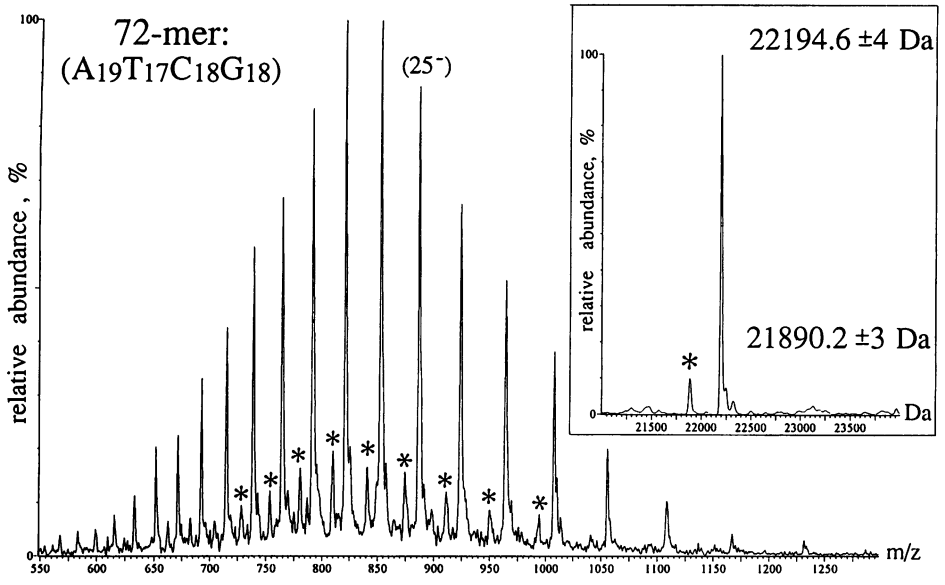

Figure 3. Multiply charged ion mass spectrum of a 72-oligomer ($A_{19}T_{17}C_{18}G_{18}$). The measured molecular mass of the major component (22194.6 ± 4 Da) corresponds to the expected structure (expected mass: 22192.1 Da). The minor series (*: measured molecular mass of 21890.2 ± 3 Da), represents a mixture of different deletion (n-1) forms which were not resolved under these conditions.

triethylammonium adducts were observed while ion pairs with sodium ions persisted at least partially. A molecular mass of 8589.8 Da was measured with an accuracy of 0.01% corresponding to the expected mass (8588.9 Da) of the 28-mer oligonucleotide in its free acid form. The resolution of the instrument was also sufficient to detect minor impurities in the preparation with measured masses of 8283.6 and 8262.4 Da. The mass differences of 306.2 and 327.4 Da correspond closely to the expected masses of a deoxythymidine T (304.2 Da) and deoxyguanosine G (329.2 Da) residue respectively. Surprisingly, there are no T or G residues at the 5′ end of the 28-mer indicating that internal deletions had occurred during automated synthesis. Mass spectrometry thus showed that internal deletions may occur to a minor extent in synthetic oligonucleotides although the automated synthesis cycle included an endcapping step with acetic acid anhydride after each coupling cycle. An n-1 form was also observed by capillary gel electrophoresis (16) but was thus far interpreted as a single product lacking the 5′-terminal deoxycytosine.

Desalting procedure for large oligonucleotides (> 10 kDa). Up to 10 kDa, simple addition of an ammonium base to the sample solution was generally sufficient to obtain high resolution spectra thus facilitating the measurement of accurate molecular weights. In order to extend the applications of ESI-MS to larger oligonucleotides, sample pretreatment according to Stults and Masters (5) was applied with minor modifications which allowed the measurement of the molecular mass of oligonucleotides with over 100 nucleotides (see Materials and Methods). Figure 2 demonstrates the efficiency of this desalting procedure. Analysis of a 40-mer dissolved in H_2O/CH_3CN (50/50), 1% TEA resulted in broad peaks because of unresolved sodium adducts which lead to a shift of the different charge states to higher average m/z values making accurate mass determination very difficult. Furthermore, sensitivity is affected by the presence of salts as can be judged from the relative heights of signals compared with the internal standard TpTpT (m/z = 849.2). It appeared that under these conditions, efficient desolvation was limited so that a signal was only observed at the beginning and at the end of the injection where dilution with the eluant (20 μM TpTpT in aqueous 50% acetonitrile, 1% TEA) occurred. On the contrary, the spectrum of another 40-mer obtained after two treatments with 10 M ammonium acetate followed by precipitations with cold ethanol was greatly improved and led to a precise measure of its molecular mass (Figure 2b; measured mass: 12317 ± 3 Da, expected mass: 12316.3 Da).

The mass spectrum of a 72-mer obtained after three treatments with 10 M ammonium acetate followed by three precipitations with cold ethanol further supported the above results (Figure 3). The measured molecular mass of 22195 ± 4 Da corresponds very closely to the expected mass (22192.1 Da). No sodium adducts were observed showing the efficiency of this desalting procedure. A minor series of multiply charged ions was also detected, indicating the presence of deletion or truncation products, since the measured mass difference (304.4 Da) of these two components was close to the mass of a single nucleotide. However, as the resolution of the instrument was insufficient to clearly resolve these minor multiply charged peaks, it was not possible to define unambiguously which nucleotide was lacking. Since these impurities are most likely the result of incomplete reactions during automated synthesis, it would be of interest to apply more powerful separation methods to analyze them separately by ESI-MS. To this end, anion-exchange HPLC on non-porous, small diameter particles is presently being evaluated.

Up to 25 kDa, an intensive removal of sodium ions through a treatment with 10 M ammonium acetate allowed accurate mass measurements by ESI-MS and detection of (n−1) forms. In most cases, the precision of the measurement was sufficient to specify which nucleotide was lacking. In order to extend the range of ESI-MS up to the range of even the largest synthetic oligonucleotides, a 132-mer was thoroughly desalted by five precipitations with cold ethanol following treatments with 10 M

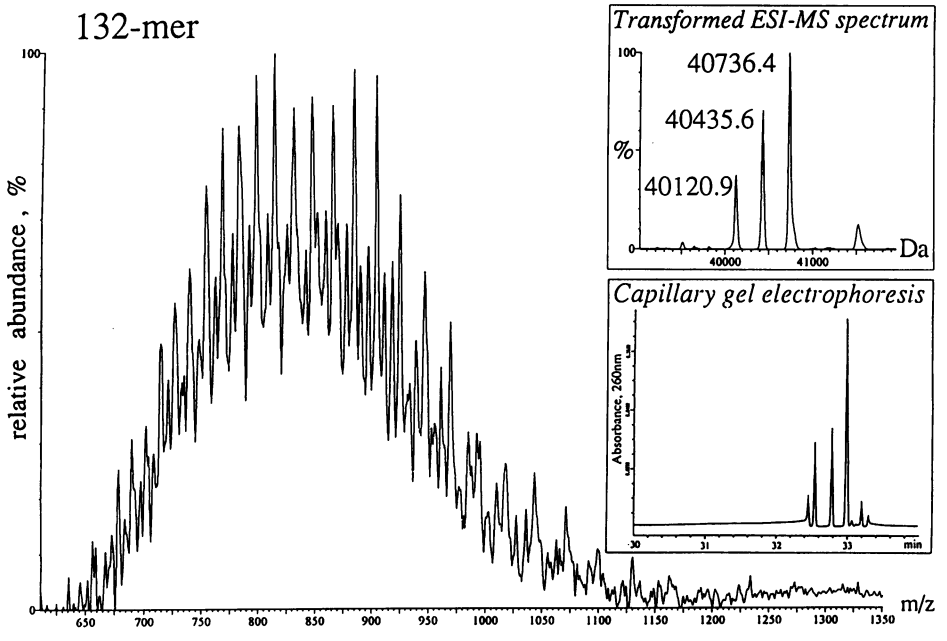

Figure 4. Characterization of a synthetic 132-mer by negative ESI-MS (expected mass: 40722.4 Da) and capillary gel electrophoresis. The multiply charged ion mass spectrum was obtained after five treatments with 10 M ammonium acetate and precipitations with 80% cold ethanol. The real mass scale spectrum and the capillary gel electropherogram of this 132-mer are shown to indicate that the sample was significantly contaminated with at least three other products which may not have been completely resolved by the mass analyzer thus explaining the low signal−noise ratio. The differences between the observed and the expected masses are probably due to a not complete removal of sodium after treatment with 10 M ammonium acetate.

ammonium acetate (Figure 4). The first overview of the spectrum reveals at least three series of partially resolved peaks and a significant background noise. A more precise examination of the peak widths shows that they are rather sharp (<3 m/z units) and that there is room for other peaks between the consecutive multiply charged ions of one series. Capillary gel electrophoresis further indicated that the 132-mer preparation was actually a mixture of at least four products with the expected oligonucleotide representing only 50% of the total quantity (see Figure 4). A comparable result has been obtained after treating the mass spectrum with the Maximum Entropy algorithm. Three components had mass differences close to the masses of single nucleotides (40736 ± 16 Da; 40436 ± 17 Da and 40121 ± 22 Da) indicating the presence of n−1 and n−2 forms. This is not surprising considering the difficulty of synthesizing such a large oligonucleotide. At the present time, it is not possible to define which nucleotides were missing, since relative standard deviations were too large. These results show however, that the actual limit of ESI-MS for oligonucleotides analysis is close to the limit of the synthetic capabilities of present day automatic synthesizers. ESI-MS thus represents a general method to analyze synthetic oligonucleotides.

Relative quantification of oligonucleotides by ESI-MS and capillary gel electrophoresis

As shown above, internal deletions may occur during the automated synthesis of oligonucleotides. These contaminating n−1 and n−2 forms can be observed by capillary gel electrophoresis but the missing nucleotide is hard to identify. On the contrary, ESI-MS allowed in most cases to define which nucleotide was lacking. It was thus of interest to evaluate whether ESI-MS could also provide quantitative data on the relative amount of contaminating oligonucleotides in a preparation since their physical and chemical properties are quite related. In order to answer this question, mixtures of synthetic 27- and 28-mers were analyzed by ESI-MS in the negative ionization mode. Figure 5

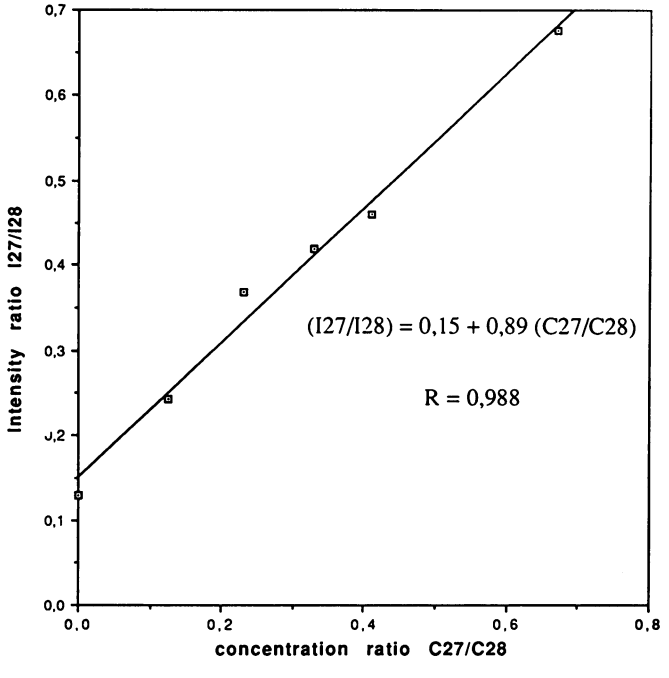

Figure 5. Relative dependence of the mass intensity ratios of identically charged ions on the concentration ratios of synthetic 27- and 28-mer oligonucleotides.

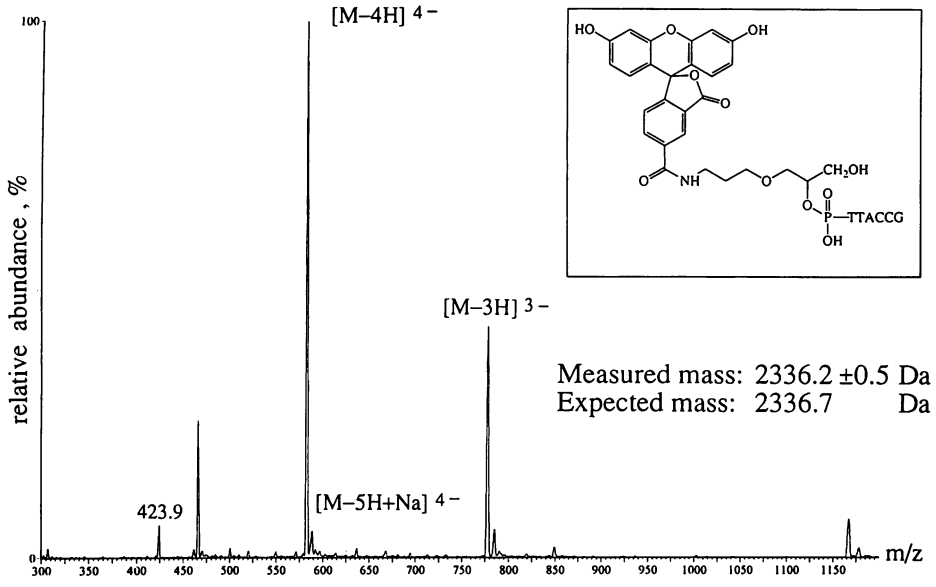

Figure 6. Verification by negative ESI-MS of the labelling of the synthetic oligonucleotide TTACCG with FAM amidite.

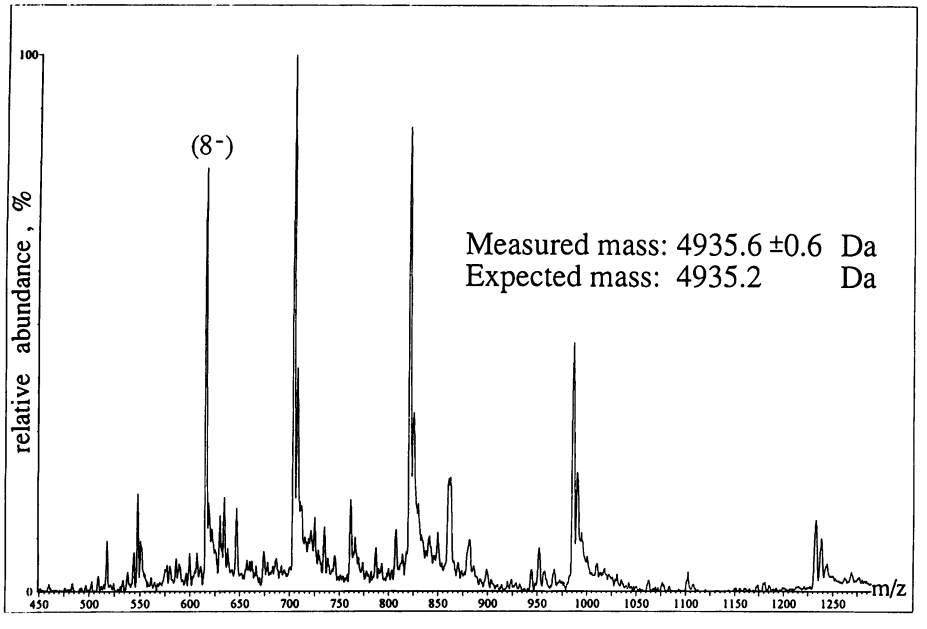

Figure 7. Negative ESI-MS of a KDEL peptide sequence coupled to a 12-mer oligonucleotide.

represents the intensity ratios of multiply charged ions of identical charge states for different concentration ratios of the 27- and 28-mers (see Materials and Methods). Concentration ratios from 0 to 0.670 were investigated since most contaminating products are present in low relative amounts. A fairly good correlation between ion intensity and concentration ratio was observed with a correlation coefficient of 0.988 indicating that quantitative answers can be obtained. However, care must be taken to employ identical measurement conditions to obtain comparative results.

ESI-MS of chemically modified oligonucleotides

The high accuracy of mass measurements on oligonucleotides makes ESI-MS a powerful tool to detect and characterize modified synthetic oligonucleotides. Accurate molecular mass determinations are at present the most rapid way to verify such modifications as the derivatization of oligonucleotides with fluorescent markers or the introduction of thiophosphate groups. The following examples are shown to highlight some of these applications.

Labelling of the synthetic oligonucleotide TTACCG with the fluorescent marker FAM amidite could be confirmed as shown in Figure 6, since the measured molecular mass of 2336.2 ± 0.5 Da corresponded closely to the expected value (2336.7 Da). The high accuracy of the measurement allowed also to verify that the fluorescent label was still present in its lactone form despite the basic character of the sample solution (1% TEA in aqueous 50%

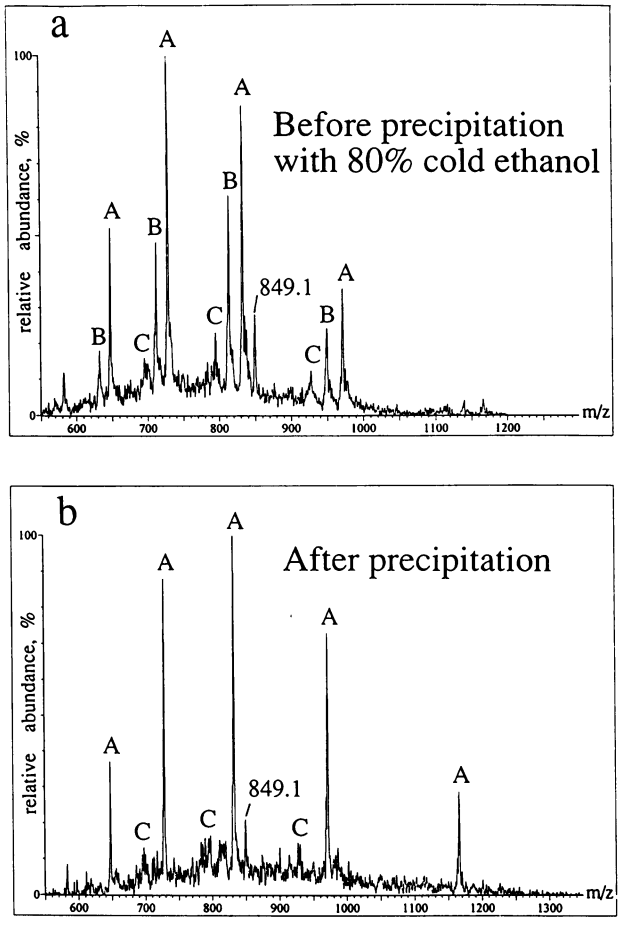

Figure 8. Multiply charged ion mass spectrum of a phosphorothioate 18-mer oligonucleotide dissolved in H_2O/CH_3CN, 1%TEA before (**a**) and after (**b**) precipitation with 80% cold ethanol in the presence of 10 M ammonium acetate.

acetonitrile solution). The peak at m/z = 423.9 corresponds to the doubly charged ion of the internal standard TpTpT (molecular mass: 850.2 Da).

Controlling the expression of viral genes or oncogenes with antisense oligonucleotides has received considerable attention due to the therapeutic potential of such an approach (17,18). As drug candidates for therapeutic use in humans, these compounds have to undergo the same rigorous quality control as for example recombinant proteins. In this respect, ESI-MS may play an important role in the future providing that high quality spectra can be obtained routinely. In order to enhance their *in vivo* activity, antisense oligonucleotides may be covalently linked to peptides with defined functions. An accurate measurement of the molecular mass of such conjugates is thus crucial to confirm the structure of both the oligonucleotide and the peptide components and to estimate the purity of the product after the coupling reaction. Figure 7 shows the spectrum of an oligonucleotide–peptide conjugate containing a KDEL signal sequence (19). The KDEL sequence is known to be retained in the endoplasmic reticulum (20). It is supposed to help these conjugates to reach the endoplasmic reticulum, from there to cross the membrane and enter the nucleus or the cytosol where the antisense oligonucleotide targets are located. A molecular mass of 4935.6 Da has been measured with an accuracy of 0.01% which agrees well with the expected mass of 4935.2 Da. Small amounts of impurities were also detected which resulted from contaminants in the original oligonucleotide used for coupling (data not shown).

Figure 8a represents the multiply charged ion mass spectrum obtained for a synthetic antisense phosphorothioate oligodeoxynucleotide. After synthesis of the 18-mer phosphorothioate, the product was purified by reverse-phase HPLC resulting in a very broad peak. Because phosphorothioate oligonucleotides have a lower solubility than their phosphodiester counterparts, analysis by capillary gel electrophoresis was also inconclusive. It was thus not possible to conclude whether the width of the HPLC peak was due to a mixture of diastereoisomers or to the presence of impurities. In order to confirm the structure and the purity of the product, a solution of phosphorothioate oligonucleotide (10 pmol/μl) was dissolved in aqueous 50% acetonitrile containing 1% TEA and analyzed by ESI-MS in the negative ionization mode. Surprisingly, three series of multiply charged ions were detected indicating that two impurities were present. The measured molecular mass of the major series (compound A) correlated well with the expected mass (5834.5 ± 1.7 Da measured, 5835.6 Da expected; the peak at m/z = 849.1 corresponds to the standard TpTpT used to optimize the spray). The two impurities B and C (molecular masses 5703.3 and 5567.4 Da) were not identified (Figure 8a). Moreover, impurity B was absent from the spectrum after precipitation with 80% (v/v) cold ethanol in the presence of 10 M ammonium acetate while impurity C persisted partially (Figure 8b). This indicated that impurity B and to a certain extent impurity C did either not precipitate or that the treatment with ammonium acetate had transformed them into compound A. The second possibility is less likely in view of their lower molecular masses.

It is thus of great importance to notice that the precipitation with cold ethanol could be selective and that care must be taken to verify first if the sample is pure or, in presence of a mixture, that no discrimination could occur during the precipitation.

Detection of ammonium adducts of oligonucleotides by positive ESI-MS

Up to this point, all data have been acquired in the negative ionization mode after dissolving the oligonucleotides in an aqueous 50% acetonitrile solution containing 1% TEA. While residual sodium adducts were detected and in spite of the large excess of amine (ammonia, TMA or TEA), no corresponding ammonium adducts with the phosphate groups were observed. By acquiring data in the positive ionization mode, it became possible to detect such species with masses corresponding to oligonucleotides with one or more ammonium adducts (Figure 9). Interestingly, only singly charged ions were detected. These spectra illustrate the effects of different amines on the trimer TpTpT, showing that TEA and TMA were more efficient in reducing the amount of sodium adducts than ammonia. The use of ammonia allowed the coexistence of both ammonium and sodium adducts, since it was possible to detect ions with one ammonium (m/z = 868.2), one sodium (m/z = 873.3), and ions with both one sodium and one ammonium adduct (m/z = 889.1). It is thus likely that the ammonium ions form ion pairs with the phosphate groups of the oligonucleotide by displacing sodium ions. It is presently unclear where the excess positive charge resides, one possibility being that thymidine itself is protonated. Further studies will be needed to elucidate this point. Because

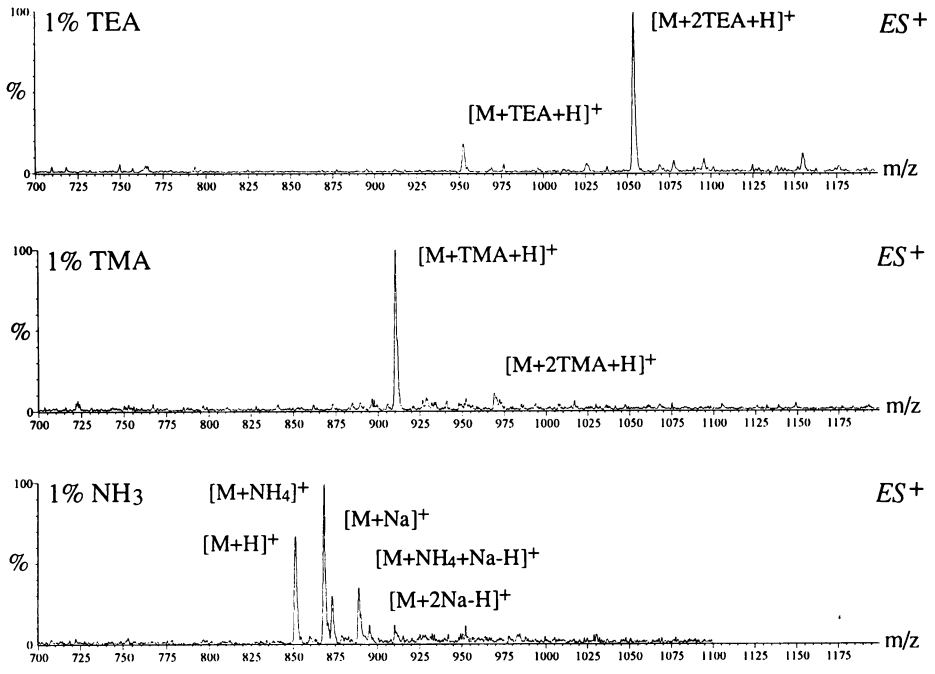

Figure 9. Observation of ammonium adducts of the trimer TpTpT in positive ESI-MS by adding different bases (ammonia, TMA and TEA) to the preparation, showing that ammonia is the least efficient in reducing the amount of sodium adducts.

of the low sensitivity in the positive ionization mode, it was not possible to perform MS/MS experiments to localize the position of the positive charge. By increasing the cone voltage from Vc = 40 V to Vc = 120 V, ions in the interface were accelerated to such a point that collisions with residual gas molecules led to a decrease in the number of ammonium adducts, leading finally to the oligonucleotide with no more adduct (data not shown). However, the detected sodium adducts (in the case of ammonia) were not affected by an increased Vc indicating that the interactions between sodium ions and the phosphate groups were significantly stronger. Furthermore, the measurements showed that the value of Vc necessary to destroy all ammonium adducts increased with the basicity of the amine bases (ammonia < TMA < TEA), confirming the choice to use TEA for negative ESI-MS on larger oligonucleotides. Competition experiments between the different amines showed finally that TEA had the highest tendency to form adducts with TpTpT in agreement with its tendency to form primarily di-adducts (see Figure 9). Similar results were obtained with the synthetic 6-mer (TTACCG) but it became more and more difficult to produce positive ions for the larger oligonucleotides since it is no longer possible to neutralize all the negative charges on the phosphate groups either with a proton or with an ammonium salt.

DISCUSSION

In this report, it has been shown that ESI-MS allows measurement of the molecular mass of synthetic oligonucleotides quickly and precisely up to at least 72 nucleotides. This agrees well with the capabilities of present day automatic synthesizers, since 90% of the synthesized oligonucleotides are smaller than 100 nucleotides. The results obtained on a 132-mer show that the actual limit of ESI-MS for oligonucleotide analysis is not only linked to the limits of the mass spectrometer but also to the purity of such large synthetic molecules. The observed peak width (see Results) shows that it would probably be possible to resolve multiply charged ions from an oligonucleotide of 200 residues if such a synthetic oligonucleotide would be chemically pure. The purity is the real limitation to the extention to larger oligonucleotides. Therefore we believe that efforts should be made to develop purification methods able to separate large oligonucleotides from their n−1, n−2, n−x forms which could then be analyzed separately by ESI-MS. A separate mass measurement of these deletion forms would also allow an increase in the mass analyzer resolution due to better sensitivity, so that the lacking nucleotides could be defined. However, for oligonucleotides larger than 10 kDa, it will become increasingly difficult to separate components with mass differences smaller than 15 Da because of their natural isotopic distribution.

Quantification of oligonucleotides was performed by ESI-MS in order to study whether this methodology could provide data for evaluating the relative amount of contaminating deletion forms produced during their automatic chemical synthesis. The obtained proportionality between the mass intensity ratios and the concentration ratios shows that the response factors for two identical charged states are similar. This is not surprising considering the fact that the contaminants differed from the major oligonucleotide by only one nucleotide. However, whether these results can be extended to other oligonucleotides will have to await more extensive studies.

Analyses of oligonucleotides by positive ESI-MS were interesting as they provided direct evidence that an excess of ammonium ions could displace the tightly bound sodium adducts on the phosphate groups, whereas desalting by reverse-phase HPLC was not sufficient. Surprising was the detection of any ions in the positive ionization mode when the oligonucleotide was dissolved in a basic solution (1% amine base). Care had been taken to employ identical measurement conditions so that the

single difference between analyses in the positive and negative ionization mode, was due to the polarity of the applied voltages. One explanation of this phenomenon could be that most species have actually carried an excess of negative charge but that ESI-MS is sensitive enough to detect the few percent of oligonucleotides with an excess of positive charge which are present in the spray. This hypothesis is supported by the fact that the positive total ion current (TIC) is actually very low compared with that obtained in the negative ionization mode. Such a phenomenon has already been observed by Kelly (21) who compared analyses of proteins in the positive and negative ionization mode, and suggested that the electric field played an important role.

In conclusion, the present study has shown that ESI-MS is a powerful analytical tool to characterize synthetic oligonucleotides giving a detailed insight into their identity and purity. Future investigations will concentrate on extending the above results to the study of noncovalent interactions between oligonucleotides and small molecules such as metal ions and cationic lipids.

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