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Characterization of synthetic oligonucleotides containing biologically important modified bases by MALDI-TOF mass spectrometry

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Abstract

Oligonucleotides containing modified bases are commonly used for biochemical and biophysical studies to assess the impact of specific types of structural damage on DNA structure and function. In contrast to the synthesis of oligonucleotides with normal DNA bases, oligonucleotide synthesis with modified bases often requires modified synthetic or deprotection conditions. Furthermore, several modified bases of biological interest are prone to further damage during synthesis and oligonucleotide isolation. In this paper, we describe the application of MALDI-TOF-MS to the characterization of a series of modified synthetic oligonucleotides. The potential for and limits in obtaining high mass accuracy for confirming oligonucleotide composition are discussed. Examination of the isotope cluster is also proposed as a method for confirming oligonucleotide elemental composition. MALDI-TOF-MS analysis of the unpurified reaction mixture can be used to confirm synthetic sequence and to reveal potential problems during synthesis. Analysis during and after purification can yield important information on depurination and base oxidation. It can also reveal unexpected problems that can occur with non-standard synthesis, deprotection or purification strategies. Proper characterization of modified oligonucleotides is essential for the correct interpretation of experiments performed with these substrates, and MALDI-TOF-MS analysis provides a simple yet extensive method of characterization that can be used at multiple stages of oligonucleotide production and utilization.

Keywords

MALDI-TOF; base analogs; synthetic oligonucleotides; oxidative damage; isotope cluster

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Introduction

Modified bases can occur in DNA as a consequence of chemical damage as well as incorporation of base analogs, many of which are important drugs (1-20). Synthetic oligonucleotides containing modified bases are commonly used to determine the biological consequences of base substitutions and DNA damage. Although the methods for the synthesis of oligonucleotides containing the normal DNA bases are well established, the synthesis of oligonucleotides containing modified bases are generally more complicated, and modified bases are often substantially more prone to chemical change either during oligonucleotide synthesis or purification.

The accurate interpretation of experiments performed with synthetic oligonucleotides containing modified bases requires that the oligonucleotides contain the expected modifications, and that the levels and properties of potential impurities are known. Previously, several laboratories have developed GC/MS or HPLC/MS methods to examine the composition of synthetic oligonucleotides containing modified bases (21-26). However, these methods require either acid hydrolysis or enzymatic hydrolysis prior to analysis. Analysis of synthetic oligonucleotides by ^{32}P -end labeling and polyacrylamide gel electrophoresis can give a general impression of the purity. However, many modifications, including chemical damage and loss of bases from the oligonucleotide may not be revealed by electrophoretic methods.

MALDI-TOF mass spectrometry is frequently used to examine peptides (27). Less work has been done with MALDI-TOF-MS to examine oligonucleotides, and in particular oligonucleotides with non-standard bases (28-38). Indeed, Guengerich and coworkers established previously capillary electrophoresis and MALDI-TOF-MS as the standard for rigorous characterization of synthetic oligonucleotides containing carcinogen adducts (35). In this manuscript, we extend this application of MALDI-TOF-MS to the analysis of a series of synthetic oligonucleotides containing damage resulting from endogenous oxidation, hydrolysis, and halogenation. The synthesis and use of oligonucleotides containing these latter forms of DNA damage often require significant modifications in base protection and deprotection strategies, occasionally leading to unexpected product complexity. Furthermore, some of the damaged bases of biological interest are inherently unstable and undergo further modifications such as hydrolysis and oxidation during and even after careful purification and characterization by standard methods. Our results presented here illustrate many of these problems and demonstrate the value of MALDI-TOF-MS in providing characterization of synthetic oligonucleotides containing endogenous damage products and in revealing unusual, unexpected yet important potential chemical changes.

Materials and Methods

Oligonucleotide synthesis

Synthetic oligodeoxynucleotides were prepared using a Pharmacia gene assembler plus DNA synthesizer. Standard oxidation and capping solutions (39) were utilized except as indicated. The structures of the modified uracil residues used in this study, as well as the

base sequence of the 12-mer oligonucleotide containing the modified bases, are shown in Fig. 1.

Phosphoramidites were obtained from Glen Research (Sterling, VA) when possible. Methods for the preparation of oligonucleotides containing phosphoramidites for 5-chlorouracil (40), 5-formyluracil (41), 5-hydroxyuracil (14,15), 5-carboxyuracil (17), and 5-(hydroxymethyl)uracil (42) have been described previously. Standard cyanoethyl phosphoramidites were used when possible and these oligonucleotides were deprotected with concentrated aqueous ammonia (28 to 30% as NH_3) at 60°C for 12 hours. Oligonucleotides containing potentially labile bases were synthesized using labile protecting groups and deprotected with potassium carbonate in methanol (43).

Oligonucleotide purification

Following the release of the oligonucleotides from the solid-phase resin, oligonucleotides containing a dimethoxytrityl group were separated by HPLC using a Hamilton PRP-1 column and a gradient of 10 to 40% acetonitrile in 25 mM triethylammonium acetate, pH 7. Oligonucleotides were detritylated using 80% aqueous acetic acid at room temperature for 30 min and then purified using a Waters XTerra C-18 column and a gradient of 0 to 20% acetonitrile in water (44). The base composition of the oligonucleotides reported here was verified by formic acid hydrolysis followed by GC/MS analysis (7,10,14,16,21).

Oligonucleotide melting temperatures

Oligonucleotide melting temperatures and corresponding thermodynamic properties can be important indicators of the impact of specific forms of DNA damage or modification on duplex structure. The comparison of oligonucleotide melting temperatures within a series of similarly modified oligonucleotides can provide an additional indicator of oligonucleotide integrity. Oligonucleotide melting temperatures were determined by measuring the temperature-dependence of the UV spectrum of an aqueous solution of the oligonucleotide (45). The melting temperature studies of the oligonucleotides were carried out with a Cary 300 Bio UV-visible spectrophotometer and a Cary temperature controller and a 6x6 Peltier cell block (Varian, Palo Alto, CA). The temperature of the oligonucleotide solution was monitored by inserting a temperature probe into a separate cuvette containing the T_M buffer alone. The oligonucleotide was dissolved in a buffer containing 10 mM sodium phosphate, 0.1 mM EDTA, 0.1 M NaCl at pH 7.0. The extinction coefficients of the oligonucleotides were determined as previously described (46). The samples were heated from 15°C to 90 °C at a rate of 0.5°C/min and absorbance was monitored at 260 nm. After holding for 3 minutes at 90°C, the sample was then cooled to 15°C at the same rate and held constant for 10 min. The cycle was continued for five temperature ramps and the T_M values were obtained from the average of the five ramps, using the Cary WinUV Thermal application (version 3.00). The experiments were carried out at least 5 different concentrations ranging from 2 μM to 75 μM for each oligonucleotide. The hyperchromicity values are obtained from the increase in the UV absorption at 260 nm due to conversion of the DNA duplex to single strands. The average hyperchromicity at various concentrations of the oligonucleotides are reported in Table 1.

MALDI-TOF mass spectrometry

HPLC-purified oligonucleotides were desalted using size exclusion micro Bio-Spin 6 Columns (Bio Rad, Hercules, CA) as previously described (47). The columns were rinsed with 2 mL dH₂O to remove the storage buffer. Approximately 200 pmol of oligonucleotide in 20 µL dH₂O was desalted. Residual cations were further exchanged with ammonium ions by incubation with ammonium-form cation-exchange AG 50W-X8 beads (BioRad) (37). Approximately 1 µL of matrix solution (10 g/L 3-HPA and 1g/L diammonium citrate) was spotted onto a 400 µm MTP anchochip plate (Bruker) and allowed to dry at room temperature. Approximately 1 µL of oligonucleotide sample was then added onto the matrix spot and allowed to dry again at room temperature. Mass spectra were obtained with a Bruker Autoflex II MALDI-TOF mass spectrometer operated in positive ion reflectron mode.

The mass calibration standard was comprised of a series of oligodeoxynucleotides 4, 6, 8, 10, 12, and 13 bases in length synthesized in our lab. Oligonucleotide experimental masses were determined by external multi-point quadratic calibration.

Simulation of ion clusters

The mass spectrum of a synthetic oligonucleotide comprises a series of lines arising from the monoisotopic peak and higher mass peaks due to the presence of stable isotopes at natural abundance. The relative abundance of the monoisotopic peak as well as the higher mass peaks can be determined upon the basis of the molecular formula of a given molecule and the natural abundance of heavier isotopes by polynomial expansion as described previously (48-50). The molecular formulas of the oligonucleotides examined here are given in Table 1. The MALDI-TOF mass spectra obtained here appear as a series of peaks that can be simulated by a Gaussian function (51) as indicated in Equation 1.

$$f(x) = ae^{-\frac{(x-b)^2}{\sigma}} \quad (\text{Eq. 1})$$

The height of the peak, *a*, is determined by the relative abundance of a given isotopomer as determined in the polynomial expansion. The center of the peak, *b*, is the mass of that isotopomer. The mass is calculated for the monoisotopic peak, and the masses of the heavier isotopes (*M*+1, *M*+2, etc.) are obtained by adding 1 mass unit for each successive isotopomer. The value of *σ*, which determines the peak width, was empirically determined to be 0.1 to best fit the experimental spectra.

Alkaline and exonuclease oligonucleotide hydrolysis

Oligonucleotides with suspected depurination were subject to hydrolysis in concentrated aqueous ammonia. Oligonucleotide (50 pmol) in 6 µL water were mixed with 4 µL concentrated trace Metal grade ammonia (Fisher science, Tustin, CA) and heated for 5 min at 95°C. The alkali treatment cleaves the oligonucleotide backbone at the abasic site, generating a 5' fragment with a hydroxyl group and a 3' fragment with a phosphate group at the cleavage site. Following heating, ammonia and solvent were evaporated under reduced pressure. Oligonucleotides were then redissolved in 10 µL dH₂O and analyzed by MALDI-TOF-MS as above.

Partial enzymatic hydrolysis (3'→5' exonuclease digestion) was accomplished with snake venom phosphodiesterase I (Worthington Biochemical, Lakewood, NJ). Approximately 200 pmol of oligonucleotide was mixed with 25 mU phosphodiesterase, 2 μ L 10 $\times$ exonuclease buffer, and water to a total 20 μ L volume. The 10 $\times$ buffer contained 1.1 M Tris-HCl (pH 8.9), 1.1 M NaCl, 150 mM MgCl₂. The mixture was incubated at 37 °C for a desired time interval between 1.5 and 20 min. Then the pH of the mixture was adjusted to neutral by addition of 5% formic acid followed by heating for 5 min at 95°C to inactivate the enzyme. The mixture was then desalted with a BioRad micro Bio-Spin 6 column and analyzed by MALDI-TOF-MS as described above.

Results and discussion

Synthetic oligonucleotides are important for studies on the biological consequences of base substitutions and base damage

A series of synthetic oligonucleotides containing modified uracil residues were prepared using cyanoethyl phosphoramidite chemistry. The sequence of the oligonucleotides as well as the structures of the modified uracil residues incorporated are presented in Fig. 1. This series includes the 5-halogen substituted uracil derivatives as well as damaged bases resulting from the oxidation of uracil and the thymine methyl group. In this series, the modified uracil residue is paired opposite guanine. Such oligonucleotides are used for biophysical and biochemical studies including those intended to identify the substrate preferences of DNA repair enzymes. Our intent in this manuscript is to examine the use of MALDI-TOF-MS for characterization of the oligonucleotide substrates. We have identified several ways in which MALDI-TOF-MS can be used to verify the composition and sequence of the synthetic oligonucleotides as well as to identify potential chemical damage that can occur during their preparation and purification. Upon the basis of the findings reported here, we advocate the routine use of MALDI-TOF-MS for the examination of synthetic oligonucleotides containing modified bases.

Oligonucleotide mass obtained by MALDI-TOF can be used to verify the composition of synthetic oligonucleotides

In Table 1, we report the expected and experimental mass spectra for the oligonucleotides examined in this paper. The MALDI-TOF mass spectra were obtained in the positive ion and reflectron modes. Experimental masses reported in Table 1 represent the average values of at least four separate measurements and the corresponding standard deviation. Experimental monoisotopic masses were reproducibly obtained to within 0.19 Da. Differences between the average experimental mass and theoretical mass was less than 0.16 Da, or approximately 44 ppm. We note that with the Bruker standard mixture of oligonucleotides 12, 20, and 30 bases in length, differences between theoretical and experimental masses were 1 to 3 Da, demonstrating the importance of suitable calibration.

Within the series of oligonucleotides examined here, 11 of the 12 bases in each oligonucleotide were constant. The variable base, in each case, was a 5-substituted uracil derivative. As the difference between theoretical and experimental mass for the oligonucleotides reported here is less than 1 mass unit, the external calibration used here is

sufficient to distinguish the oligonucleotides from one another and to confirm that the oligonucleotide was a full-length synthetic product.

Base oxidation is a common pathway for DNA damage, both *in vivo* and under synthetic conditions. Previously, Bartolini and Johnston (31) demonstrated that MALDI-TOF-MS analysis could be used to confirm the oxidation of DNA bases within an intact oligonucleotide. In that study, laser photolysis was used to oxidize guanine with an increase in the oligonucleotide mass by 16 Da. Within the series of oligonucleotides examined here, there are four pairs of oligonucleotides that represent base oxidation events: uracil to 5-hydroxyuracil, thymine to 5-(hydroxymethyl)uracil, 5-(hydroxymethyl)uracil to 5-formyluracil, and 5-formyluracil to 5-carboxyuracil. In three of the four cases, the mass of the corresponding oligonucleotide increases by 16 Da. The oxidation of 5-(hydroxymethyl)uracil to 5-formyluracil results in a decrease in the oligonucleotide mass by 2 Da. While the change in mass of 16 Da is readily observable with oligonucleotides of this length using external calibration, verification of the oxidation of 5-hydroxymethyluracil to 5-formyluracil would likely require careful calibration.

The relative peak sizes within the ion cluster provide an additional means to verify base composition

Oligonucleotides in excess of 100 bases have been characterized by MALDI-TOF-MS (38). While the mass of such oligonucleotides generally can be measured within a few Daltons, sensitivity as well as the resolution of isotopic peaks declines. According to Koomen et al. (52) isotope-resolved mass spectra can be obtained with oligonucleotides less than 30 bases in length using reflectron mode. In Fig. 2, we show the isotope-resolved spectra for four of the oligonucleotides studied here. The lowest mass peak in the mass spectrum corresponds to oligonucleotides containing the lowest mass for each atom present in the molecule, the monoisotopic peak. Additional peaks in the isotope cluster correspond to oligonucleotides containing heavier isotopes for each atom present at natural abundance. For molecules with mass greater than the 3,000 to 4,000 Da range, the monoisotopic peak would not be the largest peak.

Presuming one could accurately determine which of the peaks in the mass spectrum is the monoisotopic peak, one could obtain the mass at higher resolution in order to confirm the base composition of the oligonucleotide. However, if the experimental mass resolution was on the order of 0.2 Da, there could be many possible combinations of the normal DNA bases that could not be distinguished upon the basis of mass alone. If one introduces the possibility that the oligonucleotide contains damaged or modified bases, the complexity of the problem increases exponentially. For molecules above 500 mass units, resolution of even 1 ppm is not sufficient to assign uniquely the atomic composition of the molecule (53).

Recent studies have shown that in addition to oxidation, DNA bases can be damaged by inflammation-mediated reactive halogen species (7,8,10) under physiological conditions. The introduction of halogen atoms into a molecule complicates the appearance of the mass spectrum due to the higher abundance of heavy isotopes of chlorine and bromine (Fig. 2C,D). However, the capacity to predict the relative peak sizes in the isotope cluster based upon the oligonucleotide composition, coupled with the profound effect of chlorine and

bromine substitution on the isotope cluster, provides a powerful method for determining the most likely composition of an unknown oligonucleotide as described below.

Simulation and comparison of the ion cluster facilitates oligonucleotide identification and quantitation

The relative abundance of the monoisotopic and heavier peaks in the mass spectrum can be calculated based upon the relative abundance of heavier isotopes and the molecular formula. For example, the peaks in the isotope cluster of the 5-bromouracil-containing oligonucleotide with the molecular formula $C_{115}H_{144}N_{46}O_{71}P_{11}Br$ could be written as shown below where a_n , b_n , c_n , d_n , and e_n refer to the isotopes present for carbon, hydrogen, nitrogen, oxygen, and bromine, respectively. Phosphorous is not included in the calculation because ^{31}P is the only stable isotope. The relative abundance of the various isotopes for each atom is represented by a normalized fraction (54):

$$\begin{aligned} & (0.98892a_0 + 0.01108a_1)^{115} \\ & \times (0.99984b_0 + 0.000156b_1)^{144} \\ & \times (0.99635c_0 + 0.00365c_1)^{46} \\ & \times (0.99759d_0 + 0.00037d_1 + 0.00204d_2)^{71} \\ & \times (0.5054e_0 + 0.4946e_1) \end{aligned}$$

When the exponential expansion is completed, the relative abundance for the monoisotopic peak is 0.100, and for the 9 peaks calculated at successively higher mass: (M+1) 0.149, (M+2) 0.222, (M+3) 0.221, (M+4) 0.159, (M+5) 0.089, (M+6) 0.041, (M+7) 0.016, (M+8) 0.006, and (M+9) 0.002, respectively.

For comparison of the experimental and simulated isotope clusters reported here, the experimental file was imported into an Excel spreadsheet as a set of mass versus intensity values. Experimental values were collected at intervals of approximately 0.02 mass units. Theoretical intensities were generated using a Gaussian function as described above.

The theoretical isotope clusters for four of the oligonucleotides examined here are compared with the experimental isotope clusters in Fig 2. The correspondence between the experimental and calculated spectra are good and demonstrate the potential utility of comparing the experimental and calculated isotope clusters to confirm the elemental composition of oligonucleotides, particularly when the presence of Br or Cl is suspected. A further advantage of the simulated spectra is for quantitation. Identifying the monoisotopic peak as well as selection of the appropriate peak for quantitation can be facilitated by calculating the relative intensities of theoretical peaks in the ion cluster.

The theoretical mass of the 12-mer containing 5-bromouracil (neutral molecule) is approximately 3,724.5 Da. A list was generated of all possible oligonucleotides comprised of the normal bases A, T, C, and G with a theoretical mass within 2 Da of the mass of the BrU-oligonucleotide. Seven possible base combinations were obtained. Of these, a 12-base oligonucleotide with the composition $A_2T_2C_2G_6$ would have a theoretical mass of 3724.7 Da. Upon the basis of the experimental mass alone, these two oligonucleotides could not be

distinguished. However, the isotope cluster can be used to distinguish them. In Fig. 2D, the predicted isotope cluster for the BrU-containing oligonucleotide is shown above the experimental isotope cluster. Above, with the dotted line, the predicted isotope cluster for the A₂T₂C₂G₆ oligonucleotide is shown and is substantially different from the experimental isotope cluster.

In Fig. 3, the normalized experimental peak intensities for each of the peaks in the cluster of the BrU-oligonucleotide are plotted versus the predicted normalized intensities. A correlation coefficient (r^2) of 0.98 is obtained. In the same figure, the normalized experimental peak intensities are plotted versus the theoretical peak intensities for the A₂T₂C₂G₆ oligonucleotide. In the latter case, there is considerable scattering of the data points, and the correlation is reduced to 0.48. We propose that the comparison of experimental isotope clusters with the theoretical clusters obtained for candidate oligonucleotides could aid considerably in identifying the composition of oligonucleotide, particularly those containing chlorination or bromination damage.

MALDI-TOF analysis can provide important information on the oligonucleotide synthesis process as well as problems that can occur following synthesis

Examination of the mass spectrum of a synthetic oligonucleotide is used to confirm the composition of the oligonucleotide once purified. However, examination of the unpurified oligonucleotide mixture can be used to verify the sequence and to indicate problems that might have occurred during synthesis. In Fig. 4A, we show the spectrum obtained from the unpurified oligonucleotide mixture following deprotection and release from the resin. Note that the bases in the oligonucleotide shown in Fig. 4 are numbered from the 3' end, opposite from the normal 5' convention, but reflecting the synthesis of oligonucleotide from the 3' end.

In routine laboratory situations, no mass spectrometry analysis of the oligonucleotide is made until after the final purification (Fig 4B). However, an examination of the unpurified mixture reveals the sequence and the size of the peaks corresponding to truncated failure sequences generated during synthesis can be used to estimate relative coupling efficiencies. In the case of the 5-hydroxyuracil oligonucleotide prepared here, coupling of the modified base was the least efficient. Previously, Guerlavais et al. (35) described the uses of MALDI-TOF-MS to monitor solid-phase oligonucleotide analysis. In that study, the oligonucleotides were still attached covalently to the solid support with protecting groups attached. Due to potential oligonucleotide damage that can occur during oligonucleotide cleavage and deprotection, we suggest routine analysis of unpurified synthetic oligonucleotides as a means of documenting the synthesis.

In standard practice, oligonucleotides are cleaved from the resin and purified by HPLC using the hydrophobic DMT group to achieve separation of full-length from failure sequences. The DMT-containing oligonucleotide is then detritylated using aqueous acetic acid. The acid is then removed under reduced pressure. Depending upon how the oligonucleotide is handled at this step, substantial depurination can occur. Depurination following synthesis can be highly problematic, and is often not observable by standard ³²P-labelling and gel electrophoresis. As shown in Fig. 4C, depurination is readily revealed by

MALDI-TOF-MS analysis. Both adenine and guanine are susceptible to depurination, particularly under acidic conditions, resulting in the loss of 117 and 133 mass units as shown in Fig. 5. The resulting abasic site can also dehydrate, yielding mass differences of 135 and 151 mass units, respectively.

Further complications can arise from the incorporation of modified bases into synthetic oligonucleotides due to the need for unusual deprotection conditions. Often, modified bases of biological interest are substantially more prone to hydrolysis than normal bases, particularly under alkaline conditions. As an example, 5-hydroxyuracil, a damage product derived from cytosine, is known to be highly susceptible to further degradation. Simon et al. (55) demonstrated that 5-hydroxyuracil in an oligonucleotide could undergo one-electron oxidation when exposed to metal salts.

Results presented here (Fig. 4D) are consistent with the lability of 5-hydroxyuracil. Even under conditions thought to optimize the stability of 5-hydroxyuracil in oligonucleotides, considerable degradation can occur. When initially purified, the only significant lines observed in the mass spectrum of the HOU-oligonucleotide correspond to the protonated molecule and its sodium adduct (Fig. 4B). However, storage and attempts to further purify the oligonucleotide by HPLC result in substantial degradation of HOU. Two products, observed in the spectrum in Fig. 4D, result from ring fragmentation (–68 Da) and rearrangement (–12 Da) as shown in Fig. 5C. These products are the urea derivative (56) and 5-hydroxyhydantoin (55). Significant degradation can occur to oligonucleotides with modified bases during purification, and this damage may not be apparent to HPLC or gel analysis. Analysis by MALDI-TOF-MS is sufficiently convenient that it can and should be incorporated at multiple steps during the preparation, purification, and use of potentially labile synthetic oligonucleotides.

Loss of purines from DNA can reduce substantially the thermodynamic stability of a DNA helix, and MALDI-TOF-MS can be used to reveal the position of depurination

Damaged bases can reduce substantially the thermodynamic stability of DNA. The detection of locally destabilized regions of DNA could be a mechanism utilized by repair enzymes to find DNA damage. The impact of specific forms of DNA damage or base substitution on duplex stability can be determined by comparing the melting temperatures of normal and modified oligonucleotides. The melting temperatures for the series of oligonucleotides examined here (Table 1) were determined by measuring the temperature dependence of UV absorption at 260 nm. Upon the basis of data presented by SantaLucia and coworkers (57), a T_M of 51.25 °C was predicted for the oligonucleotide containing the G:T mispair under conditions used here (28 μ M oligonucleotide strand concentration). Our experimental value was 50.2°C, in reasonable agreement with the predicted value.

Within the series of oligonucleotides examined here, we expected the experimental T_M 's to vary by a few degrees at most. However, occasionally, T_M 's and hyperchromicity for a given oligonucleotide preparation were unexpectedly low. As an example, the T_M determined for an oligonucleotide containing 5-iodouracil was several degrees lower than expected. Although this oligonucleotide had been prepared and purified by standard conditions, the low T_M suggested there were problems with this sample.

One of the more common forms of DNA damage for synthetic oligonucleotides as well as DNA *in vivo*, is depurination as described above. We suspected that the aberrantly low T_M of the 5-iodouracil-oligonucleotide might have resulted from depurination during the preparation or storage of the oligonucleotide. MALDI-TOF-MS analysis revealed substantial depurination (Fig 6A). We hypothesized that the guanine mispaired with the 5-iodouracil residue might be particularly prone to depurination, relative to the other guanine residues in normal Watson-Crick G:C base pairs. If true, this would be a biologically important finding.

In order to determine the position of depurination, the partially depurinated 5-iodouracil-containing oligonucleotide was hydrolyzed in concentrated ammonia. Naturally occurring abasic sites are prone to hydrolysis and elimination reactions that result in the cleavage of the phosphate backbone. MALDI-TOF-MS analysis following hydrolysis resulted in a series of fragments corresponding to the position of the initial depurination (Fig. 6B). The position of depurination could be determined from the mass of the fragments obtained. Using relative peak heights for the resulting 5' fragments, a histogram was generated that revealed the relative depurination rates for all of the guanine residues in the oligonucleotide (Fig. 6C). In order to generate this histogram, an equimolar mixture of oligonucleotides 4, 6, 8, 10, and 13 bases was prepared. The peak intensity was measured for each oligonucleotide to determine the relationship between oligonucleotide size and relative peak size (supplemental data). Upon the basis of the observed relationship, observed peak sizes (Fig. 6B) were corrected and presented as relative molar amounts (Fig. 6C).

Surprisingly, the extent of depurination of the guanine residue mispaired with 5-iodouracil was substantially less than depurination of the two properly paired guanine residues in the center of this 12-base duplex. Previously, Zhang et al. (34) used exonuclease digestion to reveal abasic sites. Here, we demonstrate that ammonia hydrolysis followed by mass spectral analysis is a simple and efficient method for examining depurination of synthetic oligonucleotides.

Oligonucleotide sequence can be determined by partial enzymatic digestion and the position of some modified bases can be revealed

Previously, the sequence of oligonucleotides could be revealed by controlled digestion with exonucleases (26,29,33,34,36). It is generally presumed that such exonucleases work only on single-stranded oligonucleotides. However, the 12-base sequences examined here are self-complementary and have T_M 's around 50 °C. The exonuclease reactions are conducted at 37 °C. As shown in Fig. 7A, the uracil-containing oligonucleotide provides an interpretable spectrum that confirms the oligonucleotide sequence. Digestion of the series of oligonucleotides containing oxidized residues with snake venom phosphodiesterase demonstrates that the oxidized pyrimidine provides a strong block to the progression of the exonuclease (Fig. 7B, C, D, and E). In the series of oligonucleotides examined here, the placement of the modified base within 4 bases of the 3' end of the oligonucleotide prevented the use of the corresponding 5'-3' exonuclease calf spleen phosphodiesterase. The similarity in the observed melting temperatures of the oligonucleotides within this series (Table 1)

suggests that the observed differences in exonuclease activity can not be attributed to differences in duplex thermal stability.

Oligonucleotides containing HmU, HOU, and CarbU appear to stop the exonuclease whereas the oligo with FoU appears to slow progression of the exonuclease. This observation that some of the 5-substituted uracil analogs slow the progression of the exonuclease was unexpected. The common feature of the oxidized 5-substituted uracil residues is their capacity to interact with the phosphate backbone and specifically the phosphate residue that is the target of the exonuclease (Fig. 8). Our previous studies have demonstrated that the 5-hydroxy group of 5-hydroxy-2'-deoxycytidine-5'-monophosphate interacts strongly with the 5' phosphate group (58). It has been demonstrated previously that large bulky adducts also slow the exonuclease (33). Although their presence might complicate sequencing of the entire oligonucleotide, the capacity of some of the oxidized lesions to block exonuclease could be exploited to reveal positions of specific damage in biological DNA samples.

A potential side reaction during oligonucleotide synthesis is revealed by MALDI-TOF-MS

The synthesis of oligonucleotides containing modified bases usually requires the development of selective protection strategies. In the case of oxidized bases, the addition of functional groups that must be selectively protected substantially increases the complexity of the synthesis. Protecting groups must be selected that are stable during synthesis conditions, but are efficiently removed following oligonucleotide synthesis. An additional problem with the synthesis of oligonucleotides containing modified bases is that the modified bases may be stable until deprotected, but then undergo additional hydrolysis or oxidation reactions. Several modified bases of biological interest are alkaline labile, requiring mild deprotection conditions. The use of phenoxyacetyl protecting groups followed by deprotection in methanolic potassium carbonate has allowed the synthesis of several labile modified bases.

Upon close examination of the mass spectra of oligonucleotides made here with phenoxyacetyl-protected phosphoramidites, an additional peak 42 mass units higher was frequently observed (Fig 9A). With external calibration against the Bruker 3-oligonucleotide standard, it was often assumed that this peak was a potassium adduct (+38 Da). However, closer examination revealed it was not. As mild-deprotection reagents are used almost exclusively for the introduction of modified bases, it was our presumption that the +42 adduct was derived from the modified base. The same +42 adduct was unexpectedly seen in control oligonucleotides made with normal DNA bases A, C, T, and G as well. Subsequent studies showed that the +42 adduct was present only when G was incorporated, and +42 was consistent with addition of an acetyl group. We therefore substituted the standard acetic anhydride with deuterium-enriched acetic anhydride as the capping reagent for oligonucleotide synthesis.

Consistent with expectation, the +42 peak was shifted to higher mass (+45, Fig. 9B). We found that substitution of acetic anhydride with phenoxyacetic anhydride (59) eliminates this artifact as shown in Fig. 9C. We subsequently learned that Greenberg and coworkers (60) had previously discovered this phenomenon. However, it is not widely recognized, and if

not controlled, could result in substantial problems with experiments using the mild deprotection strategy.

Conclusions

We demonstrate here that MALDI-TOF-MS analysis of synthetic oligonucleotides is an efficient method for characterization that can reveal a multitude of potential problems that arise during and after oligonucleotide synthesis. Routine external calibration can provide experimental masses sufficiently close to the theoretical mass to exclude products resulting from addition, loss, or substitution of nucleotides during synthesis. Higher mass resolution is possible with appropriate internal calibration, and coupled with simulation of the ion cluster, can be used to confirm the elemental composition of an oligonucleotide, particularly when halogens have been incorporated. Analysis of the unpurified oligonucleotide can be used to confirm the oligonucleotide sequence whereas post-synthetic exonuclease digestion also can be used to confirm the sequence, but further, can be used to specifically reveal the position of some modified bases. Finally, due to the complexity of chemical reactions that can occur during sample preparation, including depurination, base oxidation, and base hydrolysis, MALDI-TOF-MS analysis should be incorporated into multiple steps of synthetic oligonucleotide preparation and use. The proper interpretation of biophysical or biochemical studies conducted with synthetic oligonucleotides requires a high level of confidence regarding the composition and purity of the oligonucleotide substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MALDI-TOF-MS	Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometry
GC/MS	Gas Chromatography/Mass Spectrometry
HPLC	High Performance Liquid Chromatography
PRP	Polymeric Reverse Phase
EDTA	Ethylenediaminetetraacetic acid
HPA	3-Hydroxypicolinic acid
U	Uracil
T	Thymine
FU	5-Fluorouracil

CIU	5-Chlorouracil
BrU	5-Bromouracil
IU	5-Iodouracil
FoU	5-Formyluracil
HOU	5-Hydroxyuracil
CarbU	5-Carboxyuracil
HmU	5-(Hydroxymethyl)uracil
T_M	Melting temperature

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Fig. 1. Oligonucleotide sequence and structures of the modified bases where R = -H, -CH₃, -F, -Cl, -Br, -I, -OH, -CH₂OH, -CHO, -CO₂H

Fig. 2. Theoretical isotopic distribution overlaps experimental confirming molecular formula of oligonucleotides

The experimental isotopic distributions of the oligonucleotides with the modified bases (lower line) were compared with a computer generated simulation of the isotopic distribution (upper line). The oligonucleotide composition was used to generate the simulated isotopic distribution. The theoretical and experimental profiles overlapped substantially, so the simulated peaks were raised from the baseline to distinguish the two. Calculated and experimental isotope clusters for oligonucleotides of the sequences TCCGGCGCXGGA are shown where **X** represents the modified bases A). Uracil (U), B). 5-

Fluorouracil (FU), C). 5-Chlorouracil (ClU), and D). 5-Bromouracil (BrU). In D), two theoretical isotope clusters are displayed. The solid calculated line above the experimental line corresponds to the BrU-containing oligonucleotide. The dotted calculated line corresponds to the isotope cluster for an $A_2T_2C_2G_6$ oligonucleotide differing in mass from the BrU-oligonucleotide by less than 1 Da.

Fig. 3. Comparison of theoretical and experimental isotope cluster peak sizes

Experimental isotope cluster peak sizes are plotted versus the normalized theoretical isotope cluster peak sizes for the 5-bromouracil-containing oligonucleotide (solid circles), and the theoretical isotope cluster peak sizes for an oligonucleotide of the same nominal mass (± 1 Da), but composed of the normal bases $A_2T_2C_2G_6$.

Fig. 4. Experimental mass spectra for unpurified HOU-containing oligonucleotide, purified oligonucleotide, and oligonucleotides damaged by depurination and oxidation

A). Oligo sequence can be read from the crude DMT-off 12mer-HOU with the failure sequence ladder. Note that the numbering of the sequence is from the 3' end, opposite from the standard 5' numbering, but reflecting the direction of synthesis. B). Pure 12mer-HOU. C). Depurination of the oligo was detected by MALDI-TOF-MS. Peak 1: 12mer with HOU; peak 2: 12mer with HOU minus A; peak 3: 12mer with HOU minus G; peak 4: 12mer with HOU minus G minus H₂O; peak 5: 12mer with HOU minus G minus A; peak 6: 12mer with

HOU minus G minus A minus H₂O; peak 7: 12mer with HOU minus G minus A minus 2H₂O. D). Oxidation product of 12mer-HOU was detected by MALDI-MS. The peak that is 12 Da smaller than 12mer-HOU corresponds to 5-hydroxyhydantoin. The -68 Da peak corresponds to the ring-fragmented urea derivative (Fig. 5)

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Fig. 5. Depurination pathways and HOU oxidation products for HOU-containing oligonucleotides

A) loss of adenine, B) loss of guanine, and C) 5-hydroxyuracil residue and its oxidation products, 5-hydroxyhydantoin and the ring-fragmented urea derivative.

Fig. 6. Location of abasic sites as revealed by ammonia treatment followed by MALDI-TOF-MS
 A). Depurination of 12mer-IU (5'-TCCGGCGC(IU)GGA-3') was observed in the MALDI-TOF spectrum. B). Backbone of depurinated 12mer-IU was alkali cleaved at abasic sites, indicating that the depurination occurred before the MALDI-TOF-MS analysis. The corresponding fragments indicate where depurination occurred. C). Histogram shows that depurination occurred at all five guanines in the sequence, mostly at the G-7 position. The guanine opposite the 5-iodouracil (G₄) is indicated with an asterisk (*). The experimental

peaks sizes in B) were corrected to molar amounts C) by using an empirically determined relationship between oligonucleotide mass and peak intensity.

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Fig. 7. Sequencing of oligonucleotides by exonuclease hydrolysis

A). 3'-exonuclease was used to sequence the oligo 12mer-U (5'-TCCGGCGCUGGA-3'), and the sequence was deduced from the mass difference between neighboring peaks in the MALDI spectrum. Oligo sequence can be read from exonuclease digested fragment ladder; -A, -G, -C, and -T represent loss of the corresponding mononucleotide unit. However, some modified bases are resistant to 3'-exonuclease cleavage. The 3'-exonuclease activity was completely blocked at CarbU (E), and significantly slowed at HmU (B) and HOU (C), and slightly slowed at FoU (D). Here, spectra A and D were obtained with 200 pmol oligo/

25mU 3'-exonuclease after a 2 min digestion, and spectra B, C, and E were obtained after 5 min digestion. Numbers above the peaks correspond to the length of the oligonucleotide.

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Fig. 8. Proposed interaction between 5-substituent and internucleotide phosphate linkage that could account for the inhibition of exonuclease activity

Fig. 9. Formation of an acetylguanine artifact during oligonucleotide synthesis using phenoxyacetyl mild deprotection reagents

(A) 12mer oligonucleotide containing normal bases (X = T, Fig. 1) with acetylguanine (+42 Da), (B) acetylguanine formed from deuterated acetic anhydride (+45 Da), (C) oligonucleotide formed with phenoxyacetic anhydride as capping reagent.

Table 1

Molecular formulae, theoretical monoisotopic mass, experimental monoisotopic mass, melting temperature (T_M) and the percentage of hyperchromicity values of the 12 mer oligos. T_M values were determined at a strand concentration of 28 uM in 100 mM NaCl.

Oligo	Molecular Formula	Theoretical Monoisotopic mass (M + H) ⁺	Experimental Monoisotopic mass (M + H) ⁺	T_M (°C)	Percentage of hyperchromicity
U	C ₁₁₅ H ₁₄₅ N ₄₆ O ₇₁ P ₁₁	3647.6336	3647.694 ± 0.059	49.1 ± 0.1	13.6 ± 2.0
T	C ₁₁₆ H ₁₄₇ N ₄₆ O ₇₁ P ₁₁	3661.6493	3661.495 ± 0.227	50.2 ± 0.1	13.1 ± 0.1
FU	C ₁₁₅ H ₁₄₄ N ₄₆ O ₇₁ P ₁₁ F	3665.6242	3665.653 ± 0.083	48.9 ± 0.4	13.1 ± 0.4
CIU	C ₁₁₅ H ₁₄₄ N ₄₆ O ₇₁ P ₁₁ Cl	3681.5947	3681.586 ± 0.061	50.4 ± 0.4	15.1 ± 0.3
BrU	C ₁₁₅ H ₁₄₄ N ₄₆ O ₇₁ P ₁₁ Br	3725.5441	3725.585 ± 0.155	50.0 ± 0.3	13.4 ± 0.3
IU	C ₁₁₅ H ₁₄₄ N ₄₆ O ₇₁ P ₁₁ I	3773.5303	3773.485 ± 0.107	49.9 ± 0.3	13.6 ± 0.1
FoU	C ₁₁₆ H ₁₄₅ N ₄₆ O ₇₂ P ₁₁	3675.6285	3675.695 ± 0.100	46.3 ± 0.2	12.4 ± 0.1
HOU	C ₁₁₅ H ₁₄₅ N ₄₆ O ₇₂ P ₁₁	3663.6286	3663.633 ± 0.081	49.2 ± 0.4	11.1 ± 0.1
CarbU	C ₁₁₆ H ₁₄₅ N ₄₆ O ₇₃ P ₁₁	3691.6235	3691.676 ± 0.185	49.7 ± 0.4	11.5 ± 0.4
HmU	C ₁₁₆ H ₁₄₇ N ₄₆ O ₇₂ P ₁₁	3677.6442	3677.665 ± 0.144	49.5 ± 0.5	13.4 ± 0.9