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International Journal of Mass Spectrometry 193 (1999) 15–27

International Journal of Mass Spectrometry

Matrix influence on the formation of positively charged oligonucleotides in matrix-assisted laser desorption/ionization mass spectrometry

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Received 13 April 1999; accepted 7 June 1999

Abstract

The ionization efficiency of various ultraviolet matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) matrices was investigated. A site of fixed positive charge was generated on an oligonucleotide by addition of a quaternary ammonium. This quaternary ammonium-tagged oligonucleotide was then used as an internal standard to probe the relative ionization capabilities of 3-hydroxypicolinic acid (3-HPA), 2′,4′,6′-trihydroxyacetophenone (THAP) and 2,5-dihydroxybenzoic acid (DHBA) in positive-ion mode. MALDI-MS analysis of equimolar mixtures of the quaternary ammonium-tagged oligonucleotide and an unmodified polythymidylic acid, dT_{12}, found that 3-HPA yielded more abundant protonated dT_{12} molecular ions than either THAP or DHBA. These results demonstrate that the low ion yields previously reported for polythymidylic acid are due to the matrix utilized and are not due to the low proton affinity of thymidine. Primary, secondary and tertiary amines were also incorporated into dT_{12} to examine the effect of these different amines on the protonation efficiency of the three matrices under investigation. Similar results were obtained, regardless of the amine-tag utilized, with protonation efficiency following the trend 3-HPA > THAP > DHBA. Consideration of the various factors that might influence the overall production of positively charged polythymidylic acid finds that it is the matrix:phosphodiester backbone interaction that might play the important role in determining the optimal MALDI-MS response. These results are a step towards understanding the matrix properties necessary for optimal production of oligonucleotide molecular ions in MALDI-MS. (Int J Mass Spectrom 193 (1999) 15–27) © 1999 Elsevier Science B.V.

Keywords: MALDI; Ionization efficiency; Polythymidylic acid; Charge tags; Proton affinity

1. Introduction

The analysis of oligonucleotides, especially deoxyribonucleic acids (DNA), by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is an area of considerable interest [1–3]. The accurate characterization of high molecular weight oligonucleotides is limited by the low molecular ion abundances for these species. Tang et al. [4] and Liu et al. [5] reported UV-MALDI-MS detection from 500- to 600-mers generated via the polymerase chain reaction (PCR). Recently, Hillenkamp and co-workers demonstrated analysis of DNA up to ~2000 bases using IR-MALDI-MS [6]. However, routine mass determination of oligonucleotides by UV-MALDI-MS is limited to ~50-mers.

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PII S1387-3806(99)00104-9

Table 1
Proton affinity (PA) values (kcal/mol) of the nucleobases, deoxynucleosides, and deoxynucleoside 5′-monophosphates

Nucleobase moiety	PA nucleobases[a]	PA deoxynucleosides[a]	PA deoxynucleoside 5′-monophosphates[b]
Guanine	229.3	238.2	237.0
Adenine	225.3	237.2	237.4
Cytosine	227.0	236.4	236.8
Thymine	210.5	226.8	224.1

[a] Nucleobase and deoxynucleoside proton affinities from [41].
[b] Deoxynucleoside 5′-monophosphate proton affinities from [35].

Although considerable effort has been expended on developing MALDI-MS as an attractive approach for the analysis of oligonucleotides and smaller nucleic acids (reviewed in [1,3]), surprisingly very little research has been performed to understand the fundamentals of the ionization process for this class of analytes [7]. By contrast, considerable research has been performed that is aimed at understanding the lack of molecular ion stability [8–16].

The current explanation for the limited molecular ion abundances of higher molecular weight oligonucleotides is that protonation of the nucleobases initiates base loss which leads to strand scission along the phosphodiester backbone [11,17]. The propensity for base protonation correlates with the known proton affinities (PAs) of the four nucleobases, with guanine and adenine bases being the most likely sites of protonation and thymine being the least likely site of protonation (Table 1). Indeed, ribonucleic acids (RNAs) and DNA with selected modifications form more stable ions upon MALDI-MS than does unmodified DNA. For example, no prompt fragment ions have been observed from poly-$(T)_n$ (polythymidylic acid) analyzed with a variety of matrices [8,14,18–21]. 7-Deaza analogs of guanosine and adenosine have been shown to result in reduced fragmentation in MALDI-MS [9,22]. 2′-Fluoro analogs of guanosine and adenosine incorporated in cycle-sequencing reactions are also more stable than the unmodified nucleosides [23].

There are presently two different mechanisms invoked to explain base protonation of oligonucleotides in MALDI-MS. The first mechanism involves transfer of a proton from the phosphate group of the internucleotide linkage to a base of relatively high proton affinity, such as guanine [17]. In the other mechanism, base protonation is due to the acidic protons available from the matrix [11,24]. For either mechanism, the proton affinities of the nucleobases play an important role in determining the eventual stability of the oligonucleotide in the gas phase.

Another important factor in the production of oligonucleotide molecular ions during MALDI-MS that has received less attention is the ionization efficiency of various matrices [19,20,25]. One reason that ionization efficiency has received less attention is because of the difficulty in separating the results of the ionization step from subsequent dissociation events. That is, the final molecular ion abundance in MALDI-MS is due to the number of ions produced during the laser pulse that have *not* fragmented, either in the MALDI source [as determined by linear time-of-flight (TOF) mass analyzers], or in the field-free region of the mass analyzer (as determined by reflectron TOF mass analyzers).

Although Chen and co-workers noted that positive- and negative-ion mode signals from polythymidylic acid were comparable in MALDI-MS with various matrices [20,21], several other groups have noted the poorer molecular ion abundance in positive- versus negative-ion mode for these homopolymers [8,14,19]. Previously, Tang et al. reported that the peak height of dT_{12} in positive-ion mode was much reduced as compared to the same analyte in negative-ion mode when 2,5-dihydroxybenzoic acid (DHBA) was utilized as a matrix [14]. In that work, the authors remarked that instrumental factors were not the likely culprits for the varying responses. They suggested

that the poor ion yield in positive-ion mode is attributable to the low proton affinity of thymidine relative to the proton affinities of the other deoxynucleosides.

To date there has not been any study aimed at comparing the relative ionization efficiencies of common UV-MALDI oligonucleotide matrices [e.g. 3-hydroxypicolinic acid (3-HPA) or trihydroxyacetophenone (THAP)] for the production of positively or negatively charged oligonucleotides. We are particularly interested in whether the poor ionization efficiencies in positive-ion mode found in previous investigations [8,14,19] are attributable to differences in matrix behavior or are representative of the lower proton affinity of thymine-containing oligonucleotides.

In a previous study regarding the production of amino acids via MALDI-MS, Phillips et al. found that the ionization efficiency did not correlate with the proton affinity of the amino acids when only single analytes are present in the sample [26]. To our knowledge, a similar study has not yet been performed with oligonucleotides, although Schneider and Chait performed an investigation on the influence of base composition on ion yield for negatively charged homopolymer oligodeoxynucleotides [19]. Reilly and co-workers investigated the mass spectral response of homopolymers of oligodeoxynucleotides analyzed with three common oligonucleotide matrices under otherwise identical conditions [27]. However, that work was performed in negative-ion mode and no conclusions regarding the ionization efficiency of homopolymer oligodeoxynucleotides in the three matrices studied were presented.

In this study, we have chosen to investigate the mass spectral behavior of dT_{12}, that would not fragment promptly, tagged with a primary, secondary, or tertiary amine or a quaternary ammonium. The various amine groups have different PAs or, in the case of the quaternary ammonium, have a permanent positive charge. These modified oligonucleotides were investigated in positive-ion mode with several common UV-MALDI matrices. These model compounds allow us to investigate the relative ionization efficiencies of several common matrices in positive-ion mode. These results show that the extent of formation of positively charged polythymidylic acid is determined by the matrix and is less influenced by the proton affinity of the thymidine residue.

2. Experimental

2.1. Oligonucleotide synthesis

Reagents for oligonucleotide synthesis were obtained from Glen Research (Sterling, VA). 5′-phosphorothioate labeled-$(Tp)_{11}T$ [denoted as ps-$(T)_{12}$] oligonucleotides were synthesized using standard phosphoramidite chemistry on 1 μmol columns using a Perkin Elmer/Applied Biosystems (Foster City, CA) Model 394 DNA/RNA synthesizer. Introduction of the phosphorothioate group was carried out using 3-ethoxy-1,2,4-dithiazolin-5-one (EDITH) for 30 s during the oxidization step of phosphorylation [28]. $dT(pT)_{11}$ [denoted as $d(T)_{12}$] was synthesized on a DuPont Coder 300 DNA synthesizer (Wilmington, DE) in the Nucleic Acid Laboratory at Arizona State University, using the protocol suggested by manufacturer. dT_{12} was purified by C_{18} oligonucleotide purification cartridges purchased from Perkin Elmer.

2.2. Modified oligonucleotides

The modified oligonucleotides were synthesized by reacting the 5′-phosphorothioate labeled oligonucleotide with an alkyl halide. Although cross linking of primary amines and *N*-hydroxysuccinimide is a fast and straightforward approach for generating amine-linked oligonucleotides [12], we chose not to utilize this approach as it can lead to the formation of an amide group which possesses a proton affinity value close to that of a primary amine (Table 2). Cross linking through the phosphorothioate group also permits the generation of either primary, secondary, and tertiary amines and quaternary ammonium by using the same synthetic route.

2-Bromoethylamine hydrobromide, (2-chloroethyl)dimethylamine hydrochloride and (2-bromoethyl) trimethylammonium bromide were purchased from Aldrich (Milwaukee, WI). (2-bromoethyl)methyl-

Table 2
Proton affinity values (kcal/mol) and pK_a's of amines representative of the amine tags used in this study

Amine	Proton affinity[a]	pK_a[b]
N-methylacetamide	212.4	···
Ethylamine	218.0	10.63
Ethanolamine	222.3	9.50
Methylethylamine	225.2	4.23
Methylaminoethanol	···	9.88
Dimethyl ethylamine	229.5	···
2-(Dimethylamino)ethanol	···	9.26

[a] Amine proton affinities from [41].
[b] Amine pK_a's from [42].

amine was synthesized from hydrogen bromide and 2-(methylamino)ethanol (Aldrich) following the procedure previously described for synthesis of 2-bromoethylamine [29]. (2-bromoethyl)methylamine was characterized by ^{1}H-NMR (nuclear magnetic resonance) in the Louisiana State University NMR facility and by fast atom bombardment mass spectrometry in the Department of Chemistry Mass Spectrometry facility at Louisiana State University. (2-bromoethyl) methylamine was purified by recrystallization in acetone prior to use. Modified oligonucleotides were synthesized by reacting the monohalogenated alkyl amine and the 5′-ps-$(T)_{12}$ in a 10:1 mole ratio [100:1 mole ratio for the (2-bromoethyl)trimethylammonium bromide:5′-ps-$(T)_{12}$ reaction] overnight at pH 7.5–8.0 buffered by a 1/10 volume of 0.1 *M* sodium phosphate/sodium hydrogen phosphate [30]. The sequences and molecular weights of the modified oligonucleotides are shown in Table 3.

Table 3
Sequences and molecular weights of amine-tagged oligonucleotides investigated in this study

	Sequence	Molecular weight
Primary amine	5′-H_2N–$(CH_2)_6$–pT_{12}	3767.45
	5′-H_2N–$(CH_2)_2$–ps–$(Tp)_{11}$T	3728.48
Secondary amine	5′-H(CH_3)N–$(CH_2)_2$–ps–$(Tp)_{11}$T	3742.50
Tertiary amine	5′-$(CH_3)_2$N–$(CH_2)_2$–ps–$(Tp)_{11}$T	3756.51
Quaternary ammonium	5′-$(CH_3)_3$N–$(CH_2)_2$–ps–$(Tp)_{11}$T	3770.53

2.3. Oligonucleotide purification

The deprotected 5′-phosphorothioate labeled oligonucleotides and the modified oligonucleotides (after synthesis) were purified using anion-exchange high-performance liquid chromatography on a Beckman (Fullerton, CA) System Gold HPLC. The buffers used for separation were 25 mM triethylamine, 20% aqueous acetonitrile solution, buffered by CO_2 to pH 6.4 (buffer A) and 1 *M* triethylamine, 20% aqueous acetonitrile solution, buffered by CO_2 to pH 7.6 (buffer B). The gradient was linear from 20% B to 60% B at 2%/min at a flow rate of 1.5 mL/min. After purification, the eluate was evaporated to dryness on a LabConco centrivap (Kansas City, MO) and then reconstituted in nanopure water prior to analysis.

2.4. Mass spectrometry

All matrices and chemicals used for the mass spectrometry studies were purchased from Aldrich. Saturated solutions of 3-HPA and DHBA were prepared in a 1:1 (v:v) water:acetonitrile solution. These matrices were desalted with ammonia-activated cation exchange beads (BioRad, Hercules CA) for 20 min or longer [17] prior to mixing with 1/10 volume of 0.1 *M* aqueous ammonium oxalate [31]. Solutions of 50 g/L THAP and 2-amino-4-methyl-5-nitropyridine (2-AMNP) in methanol were combined with 1/2 volume of 0.1 *M* aqueous ammonium citrate and used without further desalting steps [31]. Equimolar solutions (25 μM unless otherwise noted) of an amine or ammonium tagged-oligonucleotide and dT_{12} oligonucleotide were prepared. A 1 μL aliquot of the oligonucleotide mixture was added to 2 μL of the matrix solution. This mixture was spotted three times (1 μL each) on the sample plate.

The mass spectrometer used for analysis was a PerSeptive Biosystems, Inc. (Framingham, MA) Voyager linear MALDI-TOF instrument with an N_2 laser. The laser was operated near or slightly above threshold laser power density. Threshold laser power density is defined here as the point at which the ion signals of both analytes in the sample are observed and resolved. Each oligonucleotide mixture was

tested at least three times in each matrix and spectra were acquired at ~10 different spots. All spectra shown here were the average of at least 40 shots. Only the spectra without significant cation adduct peaks were used for further data interpretation.

3. Results

3.1. Quaternary ammonium-tagged oligonucleotides

To adequately study differences in the ionization efficiency of various UV-MALDI matrices, one must be able to account for the differences in the desorption efficiencies among such matrices [32,33]. The approach utilized here to normalize for differences in the desorption properties of 3-HPA, THAP, and DHBA is the addition of an internal standard of similar chemical properties to dT_{12} which yields a gas-phase ion through the desorption process alone. Therefore, we are utilizing a quaternary ammonium-tagged dT_{12} which contains a site of fixed permanent charge as our internal standard. Upon desorption, an ion for this internal standard is generated without any subsequent gas-phase mediated ionization processes—the quaternary ammonium-tagged dT_{12} is a preformed ion. Thus, by referencing the relative ion abundances of dT_{12} in 3-HPA, THAP, and DHBA to this internal standard, one can normalize the relative ion signal due to protonation (i.e. formation of $(dT_{12} + H)^+$ to desorption of the preformed ion, the quaternary ammonium-tagged dT_{12}) amongst the various matrices. The implicit assumption in this approach is that the desorption behaviors of the quaternary ammonium-tagged dT_{12} and dT_{12} do not differ appreciably [34]. Because we are assuming that the yield of the quaternary ammonium-tagged dT_{12} will provide a measure of the relative desorption capabilities of the different matrices, the ratio of dT_{12} to quaternary ammonium-tagged dT_{12} will adequately reflect the differences in ionization capabilities of the three matrices investigated.

Fig. 1 is the positive-ion mode mass spectra of an equimolar mixture of quaternary ammonium-tagged dT_{12} and dT_{12} in DHBA, THAP, and 3-HPA. As the

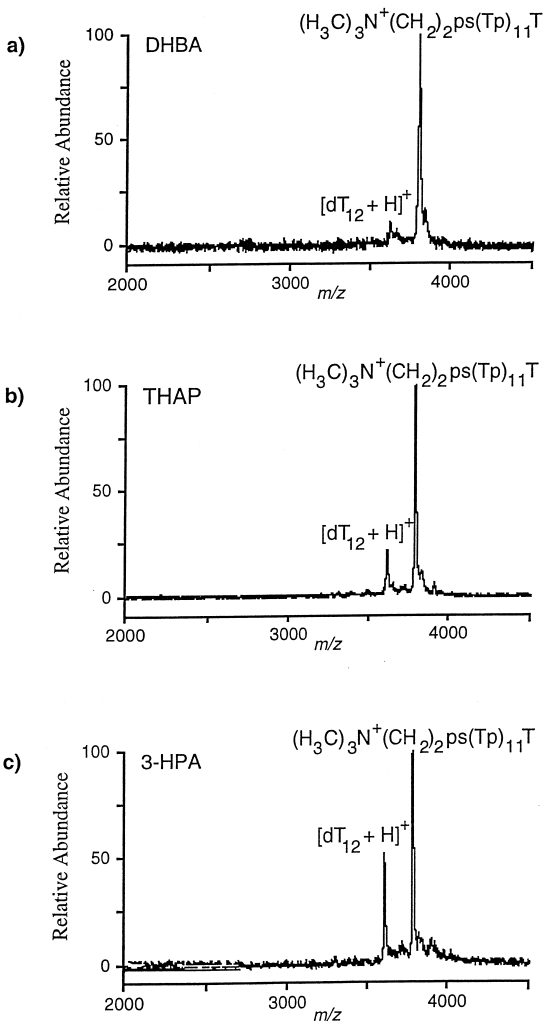

Fig. 1. Positive-ion mode mass spectra of quaternary ammonium-tagged dT_{12} and dT_{12} in (a) 2,5-dihydroxybenzoic acid, (b) 2′,4′,6′-trihydroxyacetophenone, and (c) 3-hydroxypicolinic acid. Equimolar amounts of the amine-tagged and unmodified oligonucleotide were spotted on the sample plate. Each mass spectrum is the average of 40 individual scans.

quaternary ammonium-tagged oligonucleotide contains a permanent positive charge, the ratio of the molecular ion abundance of dT_{12} to the quaternary ammonium-tagged dT_{12} provides a means of estimating the relative efficiency of protonation of dT_{12} by these different matrices. For example, if the ratio (dT_{12}/quaternary ammonium-tagged dT_{12}) is low, then dT_{12} was not protonated efficiently (more pre-

formed ions are present than are ions resulting from a gas-phase ionization event). If the ratio (dT_{12}/quaternary ammonium-tagged dT_{12}) is close to 1, then dT_{12} was protonated efficiently relative to the generation of preformed quaternary ammonium-tagged dT_{12}. As seen in Fig. 1, dT_{12} is protonated following the order of 3-HPA > THAP > DHBA.

3.2. Primary, secondary, and tertiary amine-tagged oligonucleotides

To examine whether the results seen with the quaternary ammonium-tagged oligonucleotides were atypical, equimolar mixtures of a primary, secondary, or tertiary amine-tagged dT_{12} and dT_{12} were analyzed. The proton affinities and pK_a's of the amine groups are listed in Table 2. At acidic pH values, the amine group will be protonated and thus, the primary, secondary, and tertiary amine-tagged oligonucleotides should behave similarly to the quaternary ammonium-tagged oligonucleotide when analyzed using DHBA, 3-HPA, and THAP matrices. However, as the protonation of the amine group for the primary, secondary, and tertiary amine-tagged oligonucleotides is a solution phase process (as opposed to a fixed positive charge in the case of the quaternary ammonium), the extent of positive ion formation due to the production of preformed ions versus a gas-phase protonation event may be different within the amine-tagged oligonucleotides. Moreover, the lack of the fixed positive charge in the amine-tagged oligonucleotides may result in different behavior of those oligonucleotides compared to the quaternary ammonium-tagged dT_{12}.

Fig. 2 is a plot of the ratio of the positive-ion mode molecular ion abundances of dT_{12} to the amine-tagged oligonucleotides for each of the three matrices. Evident from this plot is the similar behavior for each different amine-tag in the three matrices: 3-HPA consistently yields the highest ratio (a greater fraction of protonated dT_{12} ions are produced relative to protonated amine-tagged ions for this matrix) and DHBA consistently yields the lowest ratio, as was seen in the quaternary ammonium-tagged case. The primary, secondary, and tertiary amine-tagged investigations, however, yielded a greater percentage of

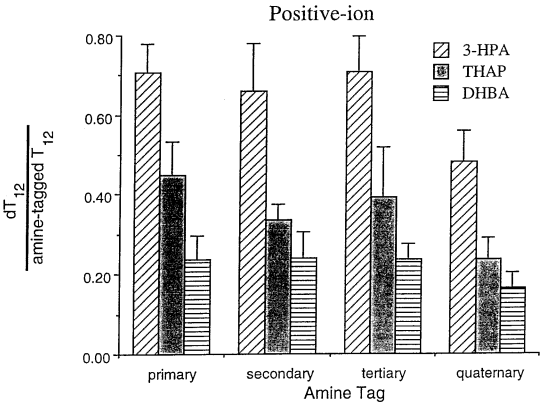

Fig. 2. Plot of the ratio of the positive-ion mode molecular ion abundances of dT_{12} to the amine-tagged oligonucleotides for each of the three matrices investigated in this study. A minimum of four separate values were averaged before plotting. The error bars are the standard deviations of the ratios. As seen, 3-HPA consistently results in the production of more protonated dT_{12} ions relative to amine-tagged dT_{12} than do any of the other two matrices.

protonated dT_{12} ions than was the case for the quaternary ammonium-tagged oligonucleotide studies (i.e. the ratios for the primary, secondary, and tertiary investigations are greater than the ratio for the quaternary ammonium). Interestingly, the secondary amine-tagged results are slightly lower in 3-HPA and THAP than for either the primary or tertiary amine-tagged results.

4. Discussion

4.1. Ion production is not intimately related to nucleoside proton affinities

There are several interesting observations relating to the results in Fig. 1. First, contrary to other published reports [8,14,19], generation of positively charged polythymidylic acid (here dT_{12}) of relatively high abundance is possible in MALDI-MS [cf. Fig. 1(c)]. The prior results of Tang et al. [14] and Schneider and Chait [19] can also be explained by this study: DHBA is the least effective matrix (of the three investigated here) at generating positively charged dT_{12} [Fig. 1(a)]. However, as seen in Fig. 1(c), when

Table 4
Proton affinity values (kcal/mol) and pK_a's of the matrices investigated in this study

Matrix	Jørgensen et al.[a]	Steenvoorden et al.[b]	Nelson et al.[c]	Burton et al.[d]	pK_a
3-HPA	214.1	···	214.5	···	3.9
2′,4′,6′-THAP	···	···	210.8	···	6–9[e]
2,5-DHBA	204 ± 3	204.2 ± 3.5	202.9	204 ± 4	2.9
2-AMNP	···	···	···	···	~10[f]

[a] [43].
[b] [44].
[c] [45].
[d] [46].
[e] Lower value estimated based on the pH of a 1 mM matrix solution. Higher value was estimated value reported in [37].
[f] Estimated based on pH of a 1 mM matrix solution.

3-HPA is utilized as a matrix, a significant positive ion signal can be produced from dT_{12}. This fact leads to the second observation: the low abundance of dT_{12} in DHBA cannot be explained simply on the basis of the lower proton affinity of thymidine. If the proton affinity of the nucleoside was the determining factor in the protonation of dT_{12}, then one would expect that the matrices investigated here would yield fairly equivalent populations of protonated dT_{12} relative to the preformed ion of the quaternary ammonium-tagged dT_{12}, as long as the thermodynamics of the protonation event (whether from the matrix or matrix-related species) were favorable.

The PAs of the nucleobases, deoxynucleosides and deoxynucleoside 5′-monophosphates and the PAs of the UV matrices investigated in this study are shown in Tables 1 and 4, respectively. Although thymidine has a lower proton affinity than the other nucleosides, the proton affinity of thymidine is still larger than the proton affinity of the ground state matrix molecules. Thus, proton transfer from a ground state matrix molecule to thymidine is thermodynamically favorable and generation of positive ions would not be prohibited in these cases.

It is worth considering the role gas-phase proton transfer reactions might exhibit on the amine-tagged oligonucleotides. As seen in Table 2, the PAs of the amine groups increase in the order primary $<$ secondary $<$ tertiary. The PA of the secondary amine group is close to the value reported for thymidine and to the recently determined value of thymidine 5′-monophosphate [35]. The PA of the tertiary amine group is higher than either thymidine or thymidine 5′-monophosphate. Thus, if protonation in the gas phase were occurring, based on the thermodynamics of the event, one would expect that thymidine would be protonated preferentially in the case of the primary amine, either thymidine or the secondary amine could be protonated, and the proton would most likely reside on the amine group in the case of the tertiary amine.

In their previous study on proton affinity effects of amino acids in MALDI-MS, Phillips et al. showed that suppression occurs when mixtures of amino acids are present with the amino acid of highest proton affinity being preferentially ionized [26]. The consistent results obtained for each matrix for the three amine-tagged oligonucleotide investigations suggest that suppression of polythymidylic acid does not occur in the presence of the amine-tagged oligonucleotide. If suppression of the analyte of lowest proton affinity in a mixture were to occur, one could make the following predictions. The ratio of protonated dT_{12} to primary amine-tagged dT_{12} would be the highest and presumably greater than one as the proton affinity of thymidine is higher than the proton affinity of the primary amine (218 versus 227 kcal/mol). The ratio of protonated dT_{12} to the secondary amine-tagged dT_{12} would be equivalent (225 versus 227 kcal/mol), and the ratio of protonated dT_{12} to the tertiary amine-tagged dT_{12} would be lowest (229.5 versus 227 kcal/mol). Furthermore, those results would occur regardless of the matrix utilized as the proton affinities of the matrices are all less than the proton affinities of even the primary amine-tagged

oligonucleotide. The consistent trend in matrix behavior seen for all three amine-tagged oligonucleotides is further evidence that ionization efficiency is more a function of the matrix than the proton affinity of the nucleosides.

In addition, the data in Fig. 2 shows that a substantial proportion of the amine-tagged oligonucleotides are most likely generated as preformed ions. A direct comparison can be made between the ratios for the quaternary ammonium-tagged dT_{12} and any of the amine-tagged dT_{12} oligonucleotides to infer the extent of preformed ions in the case of the amine-tagged oligonucleotides. The positive ion yield of dT_{12} increases about 40% when an amine-tagged internal standard is used compared to the quaternary ammonium-tagged internal standard, suggesting roughly half of the ions for the amine-tagged oligonucleotides are influenced by gas-phase events. This difference may be due to the ability of the primary, secondary, and tertiary amine tags to undergo intramolecular proton transfer with the phosphodiester backbone, or the difference could be accounted for by matrix or matrix-related ions interacting with the site of protonation. Production of a positively charged amine-tagged oligonucleotide in this case would then require an additional protonation event unlike the conditions necessary to produce a positively charged quaternary ammonium-tagged oligonucleotide.

4.2. Factors influencing the formation of positively charged ions

Although the results of Figs. 1 and 2 clearly show that formation of protonated dT_{12} from DHBA is an inefficient process, there is no immediate explanation for the different yields of dT_{12} in the three matrices studied. The current model for describing the production of oligonucleotide ions in MALDI-MS involves a secondary gas-phase ionization process that is similar in many aspects to traditional chemical ionization processes [7]. Whether it is the protonated matrix or matrix related ions that are involved in the proton transfer reaction, protonation is assumed to occur when the proton affinity of the oligonucleotide is greater than the proton affinity of the matrix or matrix related ions. As illustrated in the data in Tables 1 and 4, proton transfer reactions are thermodynamically favorable for all of the matrices investigated in this study. These results raise the important question as to what experimental factors might account for the enhanced ability of 3-HPA to generate protonated dT_{12} as opposed to the other two matrices.

There are several possible explanations for these results. One possibility is that dT_{12} does undergo dissociation in the gas phase if the exothermicity of the proton-transfer reaction is too high, as might be the case for DHBA whose proton affinity is 23 kcal/mol lower than the proton affinity of thymidine. (For comparison, the PA of THAP is 16 kcal/mol and the PA of 3-HPA is 13 kcal/mol lower than the PA of thymidine.) However, as reported by others [21,36] and found here, no lower mass prompt fragments of dT_{12} are detected in the mass spectrum. Thus, the lower molecular ion abundance of dT_{12} in DHBA is not a result of molecular ion instability, but must reflect differences in the relative abundance of protonated dT_{12} during the ionization step.

A second possibility is that the desorption efficiency of the quaternary ammonium-tagged dT_{12} oligonucleotide is different in the three matrices with DHBA being the most efficient matrix for desorption and 3-HPA being the least efficient matrix for desorption of the quaternary ammonium-tagged oligonucleotide. Alternatively, the desorption efficiency of dT_{12} is different in the three matrices investigated here.

However, as each sample spot contains an equimolar amount of quaternary ammonium-tagged dT_{12} and dT_{12}, and as the only difference between the two analytes is the presence of the charge tag, we expect that the desorption conditions for the two analytes will be comparable in each matrix. Furthermore, the fairly high reproducibility of these ion abundance ratios for all charge tags (cf. Fig. 2) strongly suggests that differential desorption of one analyte over another is not occurring to an appreciable extent.

A third possibility is that the protonation event does not occur in the gas phase but is better represented by solution phase chemistry and, thus, the pK_a's of the matrices would be a better predictor of oligonucleotide ionization. The pK_a's of each matrix

Table 5
Relative abundances of thymidine monophosphate and amine-tagged thymidine monophosphates in solution phase calculated using the pK_a values of thymidine monophosphate [47] and the amine tags (Table 2)

	$(dTp + H)^+/dTp$	$dTp/(dTp–H)^-$	$(dTp–H)^-/(dTp–2H)^{2-}$
pH = 3			
Thymidine monophosphate	···	3.8×10^{-2}	1.0×10^{-7}
Primary amine + thymidine monophosphate	1.00	···	···
Secondary amine + thymidine monophosphate	1.00	···	···
Tertiary amine + thymidine monophosphate	1.00	···	···
pH = 5			
Thymidine monophosphate	···	4.0×10^{-4}	1.0×10^{-5}
Primary amine + thymidine monophosphate	1.00	···	···
Secondary amine + thymidine monophosphate	1.00	···	···
Tertiary amine + thymidine monophosphate	1.00	···	···
pH = 7			
Thymidine monophosphate	···	4.0×10^{-6}	1.0×10^{-3}
Primary amine + thymidine monophosphate	1.00	···	···
Secondary amine + thymidine monophosphate	1.00	···	···
Tertiary amine + thymidine monophosphate	0.99	···	···
pH = 9			
Thymidine monophosphate	···	4.0×10^{-8}	9.1×10^{-2}
Primary amine + thymidine monophosphate	0.76	···	···
Secondary amine + thymidine monophosphate	0.88	···	···
Tertiary amine + thymidine monophosphate	0.65	···	···

are listed in Table 4. A pK_a for THAP has been reported in the literature to be ~9 based on estimates of aqueous solution dissociation constants considering substitutions of similar organic bases [37], but this compound produces a slightly acidic solution when dissolved in water. Thus, we estimate that the pK_a of this matrix is higher than for DHBA and 3-HPA but is most likely less than 7.

In Table 5 we have calculated the relative percentage of deprotonated thymidine monophosphate in solutions of various pH. DHBA provides the most acidic solution during sample preparation and THAP provides the least acidic solution. As seen in Table 5, as the pH of the solution increases, the relative percentage of deprotonated thymidine monophosphate will increase. Thus, one would expect that in the DHBA solution a larger population of neutral thymidine monophosphate is present than for either THAP or 3-HPA. However, under the pH conditions used in this study, one would not expect to find any protonated thymidine monophosphate in solution.

If one considers the effective charge state of the quaternary ammonium-tagged dT_{12} oligonucleotide in these same matrix solutions, then it is possible to reason that this oligonucleotide will be less negatively charged in solutions of lower pH than in solutions of higher pH. As the ammonium tag provides a fixed site of positive charge, generation of positive ions from a solution of low pH (e.g. the DHBA matrix solution has an initial pH of ~1.8 in our studies) may be easier than from solutions of higher pH (e.g. the 3-HPA matrix solution has an initial pH of ~2.8 and THAP with ammonium citrate is ~6 in our studies). To generate a positively charged ion of the quaternary ammonium-tagged dT_{12} oligonucleotide, the phosphate groups merely need to be neutralized upon transfer into the gas phase. In solutions of low pH, a greater percentage of these phosphate groups are already neutralized, thereby providing a "head start" to backbone neutralization in the gas phase.

To investigate this possibility, the quaternary ammonium-tagged dT_{12} and dT_{12} oligonucleotides were

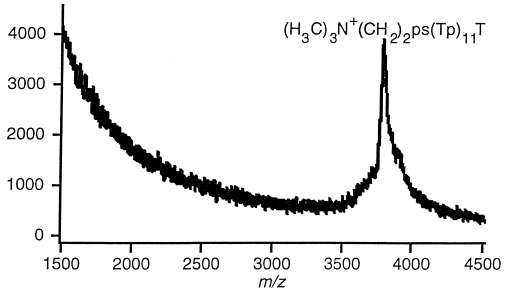

Fig. 3. Positive-ion mode mass spectrum of quaternary ammonium-tagged dT_{12} and dT_{12} in 2-amino-4-methyl-5-nitropyridine. Equimolar amounts of the amine-tagged and unmodified oligonucleotide were spotted on the sample plate. The pH of this matrix was ~7.1 prior to mixing and spotting the analyte mixture.

characterized using a basic matrix with a pK_a higher than that estimated for THAP. Fig. 3 is the mass spectrum resulting from the analysis of these analytes with 2-amino-4-methyl-5-nitropyridine. As seen in Fig. 3, in positive-ion mode there is no evidence of the formation of protonated dT_{12} in the mass spectrum. As the pH of this matrix was 7.1 prior to spotting, the fraction of neutralized dT_{12} in this matrix should be much less than the fraction of neutralized dT_{12} in a matrix of lower pH, such as DHBA (cf. Table 5). Therefore, solution phase effects can not account for the increased protonation of dT_{12} relative to the quaternary ammonium-tagged dT_{12} as found for 3-HPA.

Yet another possibility is that the number of available protons in the MALDI plume differs between the three matrices investigated. Although this possibility cannot be completely ruled out, the conditions investigated here were chosen to insure that the analyte would be the limiting reagent in any secondary gas-phase protonation event. The matrix-to-analyte molar ratios were on the order of 10 000:1 or larger. Thus, even if DHBA has 10% of the available labile protons upon desorption that 3-HPA would have available, because the thermodynamics are favorable in either case, equivalent production of protonated dT_{12} in either matrix would be predicted (i.e. the analyte is still the limiting reagent in the proton transfer reaction). Experiments at different matrix-to-analyte molar ratios (where the analyte was still the limiting reagent) were performed and no change in the relative abundance of protonated dT_{12} to the quaternary-ammonium tagged dT_{12} was found. Again, these experiments suggest that some other factor is controlling the production of protonated dT_{12} in this study. However, this possibility cannot be completely ruled out and future investigations are planned to explore the possibility that the number of labile protons in the different matrices varies considerably.

An especially intriguing possibility is that the chemical interactions (solution or gas phase) between dT_{12} and 3-HPA are substantially more favorable than between dT_{12} and DHBA. Moreover, prior results from MALDI-MS of peptide nucleic acids (PNAs) [38] and alkylated oligonucleotides [12,37] suggest that it is the phosphodiester backbone which has the most significant effect relating to the ionization efficiency of particular matrices during MALDI-MS analysis of oligonucleotides. Butler et al. investigated both oligonucleotide [3-HPA, THAP, and 6-aza-2-thiothymine (ATT)], and protein [DHBA, sinapinic acid (SA), and α-cyano-4-hydroxycinnamic acid (CHCA)] matrices for their effectiveness at analyzing PNAs [38]. PNAs differ from oligonucleotides in that the phosphodiester backbone is replaced by a peptide-like backbone. These researchers found that the protein matrices were much more effective at generating positive (or negative) ions from PNAs, and that, with the exception of ATT, the oligonucleotide matrices were ineffective for PNA analysis. Gut and coworkers investigated the applicability of a number of oligonucleotide and protein matrices, including CHCA, the methyl ester of CHCA, SA, 3-HPA, ATT, and THAP, for characterizing alkylated oligonucleotides [37]. Alkylation of the phosphodiester backbone generates a "neutralized" oligonucleotide. They found that CHCA or the methyl ester of CHCA were particularly effective matrices for analyzing charge-tagged oligonucleotides with alkylated backbones. In addition, 3-HPA was the only matrix capable of generating positive-ion data from alkylated oligonucleotides which did not contain a charge tag.

Although the alkylated oligonucleotides are similar to unmodified oligonucleotides, the neutralization of the phosphodiester backbone removes the polyanionic

character from these compounds. PNAs contain a peptide-like backbone but are still biopolymers with repeat units determined by the nucleobase at each attachment site. Thus, these two types of compounds can be considered to be hybrids between peptide-like biopolymers and oligonucleotides: each contains the standard nucleobases but the backbone is no longer polyanionic. If nucleobase interactions were an important factor in the ionization step, then PNAs and alkylated oligonucleotides could be expected to behave similarly to unmodified oligonucleotides. However, if the properties of the backbone are important factors in the ionization step, then PNAs and alkylated oligonucleotides could be expected to behave more like peptides during MALDI-MS. The results of Butler et al. [38] and Gut et al. [37] clearly show that the latter case is the dominant situation. Furthermore, Gut recently demonstrated that a depurinated alkylated oligonucleotide can be detected with enhanced sensitivity in MALDI-MS using the methyl ester of CHCA [13], lending further support to the argument that the presence of the oligonucleotide backbone rather than the nucleobases determines the appropriate type of matrix for effective MALDI-MS analysis.

It is now standard knowledge in MALDI-MS that different types of matrices are suitable for different classes of analytes [39]. That is, there are conventional peptide/protein matrices as well as conventional oligonucleotide matrices, and rarely does one find a peptide/protein matrix which is suitable for oligonucleotides and vice versa. The data obtained in this study, combined with the information from prior studies on matrix behavior, suggest that the enhanced ability of 3-HPA to form positive ions from polythymidylic acid is due to preferential interactions between the phosphodiester backbone and the matrix in either the solution phase (during crystallization) or in the gas phase (during the proton transfer reaction). DHBA is a poor matrix for generating abundant positive ions of polythymidylic acid, not because thymidine has too low of a proton affinity, but because the requisite interactions necessary for efficient proton transfer between the protonated matrix or matrix-related ions and the oligonucleotide cannot be met by this matrix.

It is worth noting that Tang et al. demonstrated that introduction of a modified thymine into polythymidylic acids allowed for the more efficient production of positively charged modified polythymidylic acid as compared to unmodified polythymidylic acid when DHBA was utilized as a matrix [14]. However, the modified thymine incorporated in those studies contained a terminal amine group which, as discussed in Sec. 3.2, is most likely protonated in solution and can be analyzed as a preformed ion in MALDI-MS. As seen in Fig. 2, amine-tagged oligonucleotides, whether the tag is attached via a terminal phosphorothioate, as is the case here, or if the tag is attached to the nucleobase, will generate more abundant positive ions than unmodified oligonucleotides. However, the ultimate yield of positively charged ions for polythymidylic acid without an additional charge tag will be determined by the matrix utilized (cf. Fig. 2).

In addition, our interpretation of the behavior of dT_{12} in DHBA versus 3-HPA does not rule out the influence proton affinity plays in overall ion production. Tang et al. have already demonstrated that even the addition of a cytidine residue to polythymidylic acids improves the positive ion abundance as compared to unadulterated polythymidylic acids [14]. From our present study, we would predict that analysis of a polythymidylic acid containing a single cytidine residue would yield more abundant positive ion yields if 3-HPA was utilized as the matrix as compared to DHBA. This prediction has been realized experimentally by Tang et al. in another work [16], although their results are complicated somewhat by additional issues of molecular ion stability.

4.3. Matrix properties necessary for positive-ion oligonucleotide analysis

The results of the present study reaffirm prior statements regarding the importance of matrix:analyte chemical interactions for optimal MALDI-MS results [7,27]. It is now well-known that the ideal matrix for oligonucleotide analysis should yield an abundant molecular ion which undergoes minimal fragmentation. 3-HPA has historically been the matrix of choice as it fulfills these two requirements. However, a

detailed explanation for the improved sensitivity resulting from MALDI-MS of oligonucleotides using 3-HPA as compared to other matrices is still lacking. While the improved ion stability found with 3-HPA is thought to be due to the lower enthalpy of protonation, proton affinity arguments are not sufficient to account for the improved initial production of molecular ions from this matrix. These initial studies demonstrate that 3-HPA is a suitable matrix for the analysis of polythymidylic acids in positive ion mode, despite the relatively low proton affinity of thymidine as compared to the other three major deoxynucleosides. Furthermore, these results suggest that it is the interaction between the matrix and phosphodiester backbone which determines the overall effectiveness of a matrix for oligonucleotide analysis via MALDI-MS.

Based on our results, it would be instructive to characterize the ability of various matrices to intercalate with oligonucleotides during crystallization to determine whether condensed-phase interactions are responsible for the improved ionization capabilities of 3-HPA relative to other matrices. Alternatively, gas-phase effects including ion yields during desorption, matrix and analyte velocities, or additional properties of protonated matrix ions (e.g. excited state proton transfer properties [40] or matrix related cluster ions) could possibly play a vital role in the overall ionization efficiency of a matrix for oligonucleotide analysis. Moreover, it will be interesting to determine whether similar trends are obtained for negatively charged oligonucleotides. These various possibilities are under investigation in our laboratory.

5. Conclusions

In this work, we have found that 3-HPA has a higher ionization efficiency in positive ion mode than THAP or DHBA during MALDI-MS analysis of oligonucleotides. The positive-ion mode results obtained in this study suggest that the efficiency of various matrices for protonating oligonucleotides is not readily explained by the differences in ground-state proton affinities between the matrix and analyte. The effect of excited state chemistry on the results shown here is unknown. The difference in proton affinities between matrix and analyte may describe oligonucleotide ion stability but, as dT_{12} does not undergo prompt fragmentation, that process should not interfere with the interpretation of the results obtained in this work. A careful consideration of the various explanations for the increased ionization efficiency of 3-HPA relative to DHBA suggests that it is the phosphodiester backbone which has a significant influence on positive ion production of oligonucleotides. Although the addition of a charge tag can improve overall ion production, matrix:oligonucleotide interactions will still be an important factor to consider when devising new approaches to MALDI-MS of unmodified oligonucleotides.

Acknowledgements

One of the authors (C.-W.C.) would like to thank Dr. Robert Hammer for use of the ABI synthesizer and for his helpful advice. She also thanks Hong Fang for his assistance in synthesizing the bromoethyl methyl amine and for his help in characterizing the final product by NMR. Financial support of this work was provided by the Department of Energy at ASU, the Louisiana Education Quality Support Fund [LEQSF (1998–1999)-RD-A-08], the National Institutes of Health (HG01777), and the Louisiana State University.

References

[1] E. Nordhoff, F. Kirpekar, P. Roepstorff, Mass Spectrom. Rev. 15 (1996) 67.
[2] E. Nordhoff, Trends Anal. Chem. 15 (1996) 240.
[3] P.A. Limbach, Mass Spectrom. Rev. 15 (1996) 297.
[4] K. Tang, N.I. Taranenko, S.L. Allman, L.Y. Chang, C.H. Chen, Rapid Commun. Mass Spectrom. 8 (1994) 727.
[5] Y.-H. Liu, J. Bai, X. Liang, D.M. Lubman, P.J. Venta, Anal. Chem. 67 (1995) 3482.
[6] S. Berkenkamp, F. Kirpekar, F. Hillenkamp, Science 281 (1998) 260.
[7] R. Zenobi, R. Knochenmuss, Mass Spectrom. Rev. 17 (1998) 337.
[8] K.J. Wu, A. Steding, C.H. Becker, Rapid Commun. Mass Spectrom. 7 (1993) 142.

[9] R. Kirpekar, E. Nordoff, K. Kristiansen, P. Roepstorff, S. Hahner, F. Hillenkamp, Rapid Commun. Mass Spectrom. 9 (1995) 525.
[10] E. Nordhoff, M. Karas, R. Cramer, S. Hahner, F. Hillenkamp, A. Lezius, F. Kirpekar, K. Kristiansen, J. Muth, C. Meier, J.W. Engels, J. Mass Spectrom. 30 (1995) 99.
[11] L. Zhu, G.R. Parr, M.C. Fitzgerald, C.M. Nelson, L.M. Smith, J. Am. Chem. Soc. 117 (1995) 6048.
[12] I.G. Gut, S. Beck, Nucl. Acids Res. 23 (1995) 1367.
[13] I.G. Gut, Int. J. Mass Spectrom. Ion Processes 169/170 (1997) 313.
[14] W. Tang, C.M. Nelson, L. Zhu, L.M. Smith, J. Am. Soc. Mass Spectrom. 8 (1997) 218.
[15] W. Tang, L. Zhu, L.M. Smith, Anal. Chem. 69 (1997) 302.
[16] W. Tang, J. Krause, L. Zhu, L.M. Smith, Int. J. Mass Spectrom. Ion Processes 169/170 (1997) 301.
[17] E. Nordhoff, R. Cramer, M. Karas, F. Hillenkamp, F. Kirpekar, K. Kristiansen, P. Roepstorff, Nucl. Acids Res. 21 (1993) 3347.
[18] G.R. Parr, M.C. Fitzgerald, L.M. Smith, Rapid Commun. Mass Spectrom. 6 (1992) 369.
[19] K. Schneider, B.T. Chait, Org. Mass Spectrom. 28 (1993) 1353.
[20] K. Tang, S.L. Allman, C.H. Chen, Rapid Commun. Mass Spectrom. 7 (1993) 943.
[21] K. Tang, S.L. Allman, R.B. Jones, C.H. Chen, S. Araghi, Rapid Commun. Mass Spectrom. 7 (1993) 63.
[22] K. Schneider, B. Chait, Nucl. Acids Res. 23 (1995) 1570.
[23] T. Ono, M. Scalf, L. Smith, Nucl. Acids Res. 25 (1997) 4581.
[24] T.A. Simmons, P.A. Limbach, J. Am. Soc. Mass Spectrom. 9 (1998) 668.
[25] T. Huth-Fehre, J.N. Gosine, K.J. Wu, C.H. Becker, Rapid Commun. Mass Spectrom. 6 (1992) 209.
[26] D.R. Phillips, M. Karas, H. Ehring, F. Hillenkamp, Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics 1993, San Francisco, CA, p. 674.
[27] N.P. Christian, L. Giver, A.D. Ellington, J.P. Reilly, Rapid Commun. Mass Spectrom. 10 (1996) 1980.
[28] Q. Xu, K. Musier-Forsyth, R.P. Hammer, G. Barany, Nucl. Acids Res. 24 (1996) 1602.
[29] F. Cortess, J. Amer. Chem. Soc. 58 (1936) 191.
[30] S. Akerfeldt, Acta Chim. Scand. 17 (1963) 329.
[31] U. Pieles, W. Zürcher, M. Schär, H.E. Moser, Nucl. Acids Res. 21 (1993) 3191.
[32] C.D. Mowry, M.V. Johnston, J. Phys. Chem. 98 (1994) 1904.
[33] M. Karas, U. Bahr, K. Strupat, F. Hillenkamp, A. Tsarbopoulos, B.N. Pramanik, Anal. Chem. 67 (1995) 675.
[34] E. Lehman, R. Knochenmuss, R. Zenobi, Rapid Commun. Mass Spectrom. 11 (1997) 1483.
[35] K.B. Green-Church, P.A. Limbach, J. Am. Soc. Mass Spectrom., submitted.
[36] E. Nordhoff, A. Ingendoh, R. Cramer, A. Overberg, B. Stahl, M. Karas, F. Hillenkamp, P.F. Crain, Rapid Commun. Mass Spectrom. 6 (1992) 771.
[37] I.G. Gut, W.A. Jeffery, D.J.C. Pappin, S. Beck, Rapid Commun. Mass Spectrom. 11 (1997) 43.
[38] J.M. Butler, P. Jiang-Baucom, M. Huang, P. Belgrader, J. Girard, Anal. Chem. 68 (1996) 3283.
[39] P.A. Limbach, Spectroscopy 13 (1998) 16.
[40] M. Gimon-Kinsel, L.M. Preston-Schaffter, G.R. Kinsel, D.H. Russell, J. Am. Chem. Soc. 119 (1997) 2534.
[41] E.P. Hunter, S.G. Lias, J. Phys. Chem. Ref. Data 27 (1998) 413.
[42] CRC Handbook of Chemistry and Physics, R.C. Weast (Ed.), CRC, Cleveland, 1977, Vol. 58.
[43] T.J.D. Jørgensen, G. Bojesen, H. Rahbek-Nielsen, Eur. Mass Spectrom. 4 (1998) 39.
[44] R.J.J.M. Steenvoorden, K. Breuker, R. Zenobi, Eur. Mass Spectrom. 3 (1997) 339.
[45] C.M. Nelson, L. Zhu, W. Tang, L.M. Smith, K. Crellin, J. Berry, J.L. Beauchamp, Proceedings of Ultrasensitive Biochemical Diagnostic 1996, San Jose, CA, p. 247.
[46] R.D. Burton, C.H. Watson, J.R. Eyler, G.L. Lang, D.H. Powell, M.Y. Avery, Rapid Commun. Mass Spectrom. 11 (1997) 443.
[47] W. Saenger, Principles of Nucleic Acid Structure, Springer Advanced Texts in Chemistry, Springer, New York, 1984.