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A matrix of 3,4-diaminobenzophenone for the analysis of oligonucleotides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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ABSTRACT

A new matrix of 3,4-diaminobenzophenone (DABP) was demonstrated to be advantageous in the analysis of oligonucleotides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. With DABP as a matrix, intact oligonucleotide ions can be readily produced with lower laser powers, resulting in better detection limits, less fragmentation and fewer alkali metal ion adducts compared with the results obtained with conventional matrices. Importantly, minimal fragmentation and fewer alkali metal ion adducts were seen even at low concentrations of oligonucleotides. It was also found that samples prepared with DABP are highly homogenous and therefore reducing the need for finding 'sweet' spots in MALDI. In addition, excellent shot-to-shot reproducibility, resolution and signal-to-noise ratio were seen with DABP as the matrix.

INTRODUCTION

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is becoming an effective analytical tool in the analysis of nucleic acids (1–10). There are still two major problems encountered in the MALDI analysis of nucleic acids despite advances in its methodology (11). One problem is the fragmentation of oligonucleitides caused by MALDI, particularly the fragmentation of large oligonucleotides. The other problem is the formation of alkali metal cation adducts, leading to decreased resolution and detection sensitivity.

The matrix plays an essential role in MALDI and thus increasing efforts have been made to identify new matrices in order to improve the MALDI performance. To date, there are few matrices that work well for oligonucleotides. 3-Hydroxypicolinic acid (3-HPA) (12) works well for oligonucleotides when used with co-matrices of picolinic acid and diammonium hydrogen citrate (DHC). Recently, it was reported that oligonucleotides of 5000–10 500 Da could be analyzed using a matrix system containing both 3-HPA and pyrazinecarboxylic acid (PCA) (13). Importantly, this matrix system allowed resolution of 23mer with a mass difference of as small as 7 Da. Quinaldic acid (QA) is also a good matrix for the analysis of DNA or RNA with <24mer when used in conjunction with ammonium citrate (14). 2,4,6-Trihydroxyacetophenone (2,4,6-THAP) is also commonly used for the analysis of oligonucleotides up to 12mer (15). It was shown that a mixture obtained by mixing equal volumes of 2,4,6-THAP acetonitrile solution and triammonium citrate aqueous solution (16) could produce $[M–H]^-$ ions rather than sodium ion adducts even in the presence of 50 mM of NaCl. 6-Aza-2-thiothymine (ATT) (17) was also used as a matrix for oligonuceotides, allowing for the detection of double-stranded DNA, particularly DNA <25mer. A detection limit of 75 fmol for 12mer DNA was reported using ATT with spermine added. Recently, it is shown that 5-methoxysalicylic acid (MSA) is a good matrix for oligonucleotides in the 12–20mer size range when spermine is used as an additive (18). The MSA/spermine co-matrix system leads to minimal formation of alkali metal ion adducts, reduced fragmentation and better resolution compared with ATT and 3-HPA with either DHC or spermine as a co-matrix (18).

Generally, a co-matrix is required for the matrices mentioned above in order to produce high-quality mass spectra with high detection sensitivity by MALDI. The overall performance of MALDI is usually not satisfactory when conventional matrices are used alone. We, recently, identified 3,4-diaminobenzophenone (DABP) as a novel matrix for the analysis of peptides and proteins by MALDI-MS (19). In the present work, we applied this new matrix to the analysis of oligonucleotides. It was observed that DABP yields better detection limits and resolution, decreased fragmentation and

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Table 1. Sequences and calculated molecular weights of the oligonucleotides examined

Segment length	Calculated mass (Da)	Sequence
6mer-1	1791.25	5′-CTTAAG-3′
6mer-2	1800.26	5′-CTAAAG-3′
12mer-1	3646.44	5′-CGCGAATTCGCG-3′
12mer-2	3655.46	5′-CGCGAAATCGCG-3′
20mer	6064.03	5′-TTACTCTGTTAATGTCCTTG-3′
23mer-1	6905.62	5′-TCCACCATTAACACCCAAAGCTA-3′
23mer-2	6912.60	5′-TCCACCATTTGCACCCAAAGCTA 3′
23mer-3	6921.62	5′-TCCACCATTAGCACCCAAAGCTA-3′
30mer	9220.12	5′-CATGTATAACCGCATTATGCTGAG TGATAT-3′

less formation of alkali metal ion adducts when compared with conventional matrices. Moreover, the prepared samples are more homogenous, resulting in excellent shot-to-shot and spot-to-spot reproducibility.

MATERIALS AND METHODS

Reagents

DABP, 3-HPA and 2,4,6-THAP were purchased from Acros Organics (NJ, USA). All oligonucleotides (HPLC grade) were synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China).

The sequences and calculated masses of oligonucleotides used in this work are listed in Table 1. In addition, $d(T)_4$, $d(T)_6$, $d(G)_6$, $d(T)_8$, $d(T)_{10}$, $d(T)_{12}$, $d(T)_{16}$ were used. All the chemicals were used as received without further purification.

Sample preparation

DABP was prepared at a concentration of 15 mg/ml in acidified methanol (methanol/HCl = 80:3, v/v). All other solutions were prepared at room temperature using deionized water from a Millipore Milli-Q system (Millford, MA, USA). 3-HPA was prepared as a saturated solution in acetonitrile/water (1:1, v/v). 2,4,6-THAP was prepared at 0.2 M concentration in acetonitrile/water (50/50, v/v). All solutions were freshly prepared daily.

TE buffer consists of 0.1M Tris and 0.01 M EDTA, adjusted to pH 7.75 with hydrochloric acid. Oligonucleotides dissolved in water or TE buffer, were further diluted with water before MALDI analysis. The matrix solution and analytes solution were mixed at an equivalent volume ratio for ~3 min and then 0.5 μl of the mixing solution was deposited on the probe directly and dried in air before the analysis.

Instrumentation

All experiments were performed in the linear mode using a Bruker Autoflex time-of-flight mass spectrometer (Bruker Company, Bremen, Germany), equipped with a nitrogen pulsed laser (337 nm). Each recorded mass spectrum consists of the sum of 30 shots at a single sampling position using the negative-ion detection mode. External calibration was used in this work.

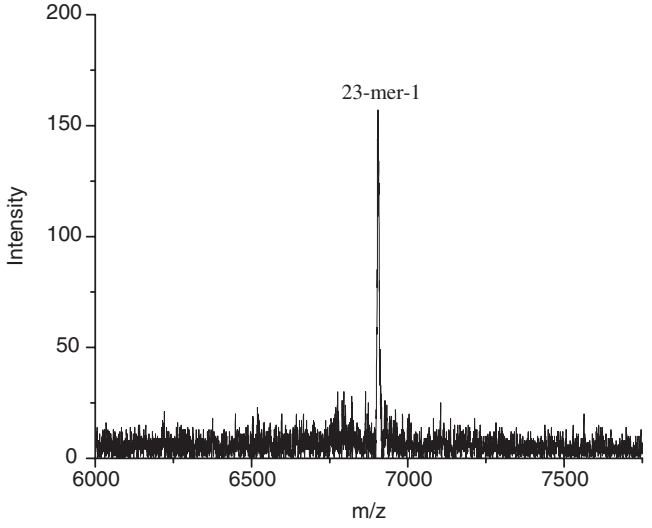

Figure 1. MALDI mass spectrum of 23mer-1 obtained using DABP as the matrix. The total quantity of this oligonucleotide loaded was 62.5 fmol.

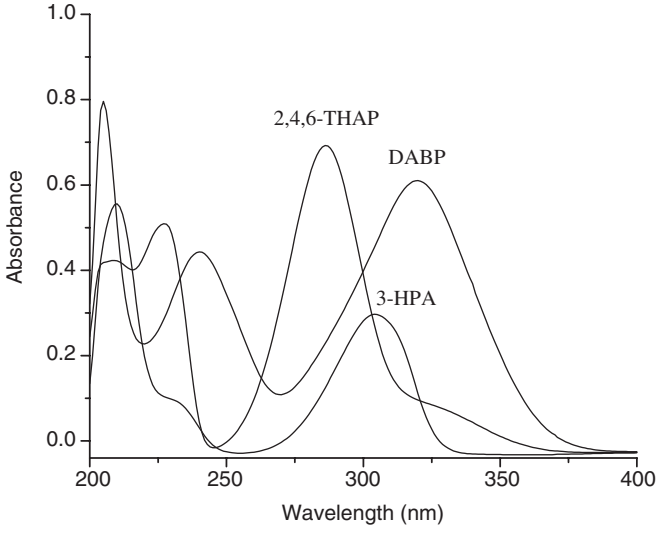

Figure 2. UV-visible spectra of the three matrices used. The concentration of each matrix was 3.0×10^{-5} M.

RESULTS AND DISCUSSION

Performance of DABP

As compared with 3-HPA and 2,4,6-THAP, use of DABP as a matrix requires lower laser powers and generates intense oligo ion signals with minimal fragmentation. Figure 1 displays a typical MALDI mass spectrum of oligonucleotide of 23mer-1. It is seen that the $[M–H]^-$ ions dominate the spectrum with few fragments and adducts, even when only 62.5 fmol was loaded. This is true at low loading which is generally difficult for conventional matrices to produce good oligo ion signals. Figure 2 shows the UV/visible absorption spectra of each of three matrices in an acidified methanol solution (methanol/HCl = 80:3, v/v). The experimentally determined the molar absorptivity of DABP was 13788 M^{-1} cm^{-1} at 337 nm, which is significantly greater than that of 3-HPA and 2,4,6-THAP. Hence, it is not

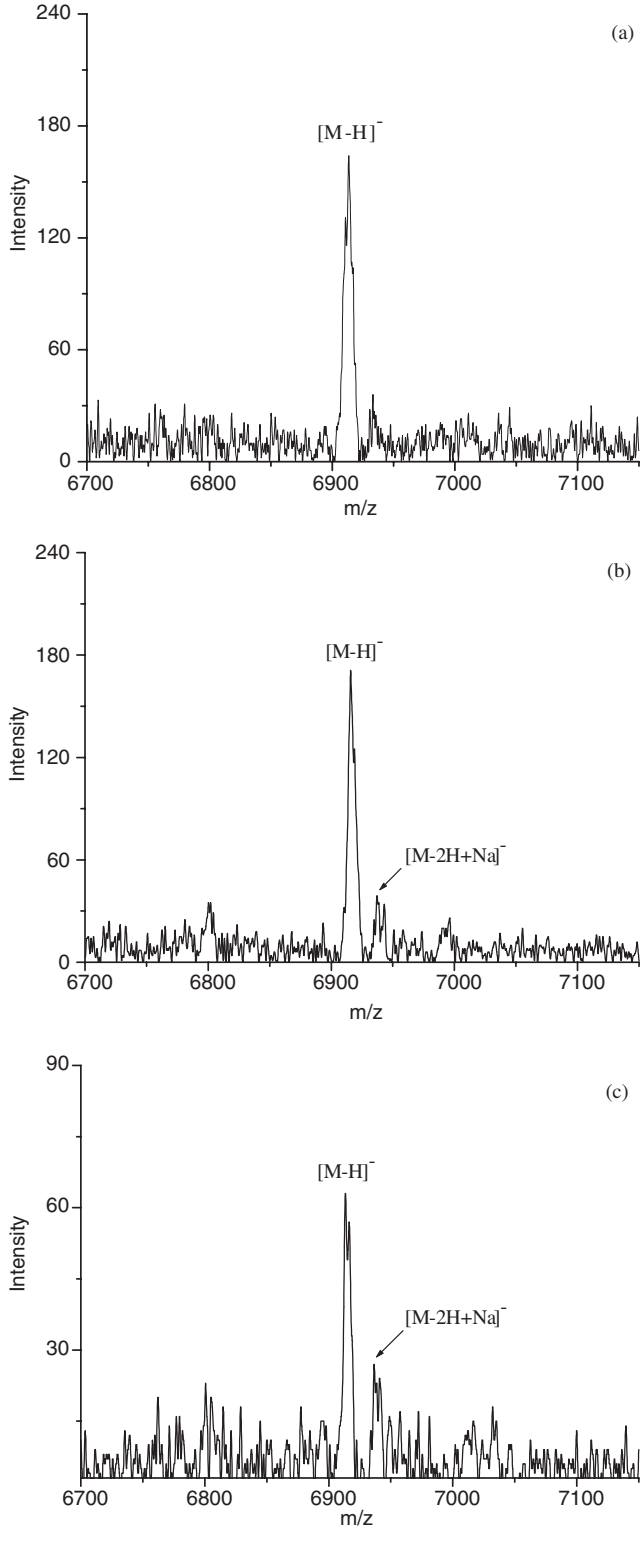

Figure 3. MALDI mass spectra of 23mer-2 (500 fmol), obtained by diluting a 10 μM oligonucleiotide stock solution with (**a**) 0.1 M, (**b**) 0.2 M and (**c**) 0.4 M of NaCl, respectively.

surprising that lower laser power is needed when DABP is used as a matrix.

Next, we tested the salt tolerance of MALDI with DABP as a matrix. It was found that the MALDI technique could

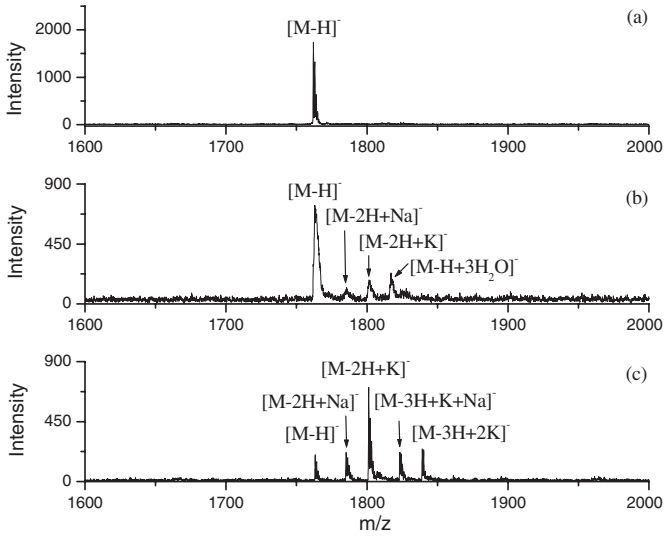

Figure 4. MALDI mass spectra of $d(T)_6$ obtained using (**a**) DABP, (**b**) 3-HPA and (**c**) 2,4,6-THAP as the matrix, respectively. The quantity of $d(T)_6$ loaded was 1.4 pmol without adding extra NaCl to the sample solution.

tolerate salts much better if DABP was used as a matrix. This was especially true when low-concentration oligonucleotides were analyzed. This test was performed by diluting the stock solution of oligonucleotide of 23mer-2 with different concentrations of NaCl solutions. The oligo stock solution was prepared at a concentration of 100 μM in TE buffer. The spectra obtained under these conditions are shown in Figure 3. The quantity of the oligonucleotide loaded onto the target is 500 fmol. In all cases, the ion signals of $[M–H]^-$ were dominant, although the ion signal intensity did decrease with an increase of NaCl concentration in sample solutions. No sodium ion adducts were clearly observed even when the amount of NaCl added to the sample solution was $\sim 5 \times 10^4$ times higher than that of oligonucleotides, as shown in Figure 3a. Although sodium ion adducts appeared at larger concentrations of NaCl, the $[M–H]^-$ ion signal of the oligonucleotide still dominates the mass spectra, as shown in Figure 3b and c. Figure 4 shows the typical mass spectra of $d(T)_6$ obtained with separately used matrices of DABP, 3-HPA and 2,4,6-THAP with no co-matrices added. It is seen that alkali metal cation adducts could not be effectively eliminated when only 3-HPA or 2,4,6-THAP were used as matrix even without extra NaCl added. In contrast, no alkali metal cation adduct peaks were observed with DABP as the matrix, suggesting that this new matrix could improve the salt tolerance in MALDI analysis of oligonucleotides.

To better compare this new matrix with other conventional matrices, literature findings and those obtained in this study are compiled in Table 2. The molar ratio of sodium chloride and oligo was used to characterize the salt tolerance of the different matrices or different methods. The molar ratio was roughly determined at the point where sodium adducts first appear in the mass spectrum. Clearly, DABP, even without any other additives or co-matrices, effectively inhibits the formation of sodium ion adducts. The other matrices or methods cannot yield comparable results under the similar conditions. Although there are many other matrices and methods that reduce the formation of the metal ion adducts

Table 2. Comparison of the salt tolerance of four different matrices

Used matrices	Additives	Sample preparation	Molar ratio of NaCl to analyte (M/M)	Sodium adducts	References
HPA	Diammonium citrate	Simple	1:4	Intense	(20)
DABA	—	Time-consuming	20:1	Few	(21)
2,4,6-THAP	Triammonium citrate	Simple	10^4:1	Few	(16)
DABP	—	Simple	10^5:1	Few	This work

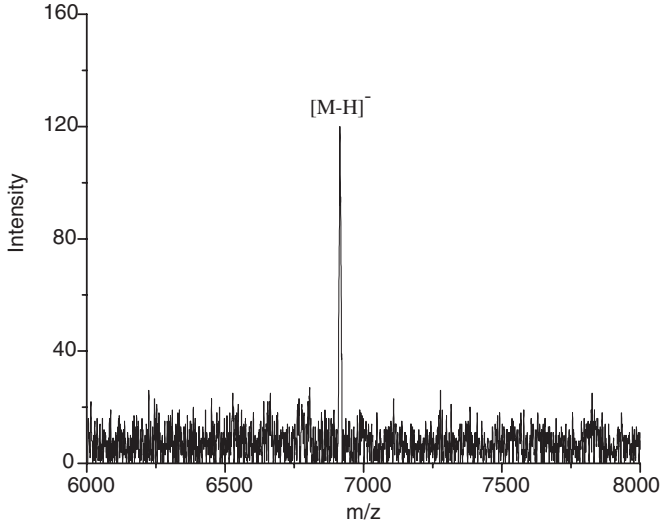

Figure 5. MALDI mass spectra of 23mer-2 (50 fmol) obtained by diluting stock solution in 1 M urea.

(11,14,18,22–30), this new matrix is unique in its ability to salt tolerance. The possible explanation for its tolerance to salt is the two amino groups in the DABP molecule, which can presumably replace alkali metal cations to bind with oligo analytes (21). As a result, DABP could function as both a MALDI matrix and a desalting reagent.

It should be noted that adducts with a gain in mass of 53 or 54 Da were sometimes observed in the MALDI mass spectra. The reason for the formation of these adducts is not clear. Furthermore, the tolerance of MALDI with other reagents such as urea was examined. Figure 5 shows the mass spectrum of 23mer-2 in the presence of 1 M urea. Clearly, there were no intense adduct peaks seen and good quality spectra were generated under this condition.

The uppermost detection limits for a number of oligonucleotides were determined using DABP as a matrix and summarized in Table 3. This new matrix improves the MALDI detection limits of oligonucleotides of <30mer. Figure 6 shows a lower limit for several of the oligos listed in Table 3. It should be noted that although a detection limit of 4 fmol was achieved for a 24mer oligo (31), great care must be taken in the sample preparation. Another protocol has been developed to isolate, concentrate and purify oligonucleotides in a microcentrifuge tube before sample analysis, allowing the analysis of oligos to a 50 nM concentration by MALDI (32). However, that protocol is too complicated and time-consuming. In contrast, the DABP matrix can readily detect a variety of oligonucleotides at lower concentrations, even without the use of any other additives or co-matrices. Importantly, the standard drop-dried procedure

Table 3. Detection limits for the oligonucleotides studied in the present work

Oligonucleotides	Detection limits
$d(T)_6$	70.0 amol
$d(G)_6$	1.25 fmol
6mer-1/6mer-2	1.25 fmol
$d(T)_{12}$	12.5 fmol
12mer-1/12mer-2	12.5 fmol
$d(T)_{16}$	31.3 fmol
20mer	31.3 fmol
23mer-1/23mer-2/23mer-3	62.5 fmol
30mer	5.00 pmol

can be utilized to prepare the sample for this matrix, improving the detection sensitivity of the MALDI technique.

It should also be noted that the use of DABP provides good resolution for low-concentration oligonucleotides that are not detected when 3-HPA or 2,4,6-THAP are used as a matrix. For example, two 12mer oligonucleotides which differ by only 9 Da were well resolved from each other, as shown by Figure 6c. The use of DABP as a matrix achieves better or comparable mass resolution compared with that obtained with conventional matrices. Figure 7 shows the typical mass spectra of $d(T)_6$ obtained with matrices of (i) DABP, (ii) 3-HPA and (iii) 2,4,6-THAP, respectively. Poorer resolution was seen for 3-HPA (Figure 7b), while the comparable mass resolution (Figure 7a and c) was seen when DABP or 2,4,6-THAP were used as matrix.

High-throughput genotyping is the main area of application of the MALDI-TOF-based nucleic acids analysis. Poor sample homogeneity has been a major problem associated with conventional matrices, limiting the throughput of the technique. In this present work, the homogeneity of samples prepared with DABP was also studied. MALDI samples using DABP matrix were found to be significantly more homogenous compared with those prepared with 3-HPA. Much better shot-to-shot, spot-to-spot and sample-to-sample reproducibility was observed with DABP as compared with 3-HPA or 2,4,6-THAP. High-quality spectra could be produced across the entire sample. The DABP sample was of such high homogeneity that no effort was needed to search for so-called 'sweet spots' to produce high-quality spectra. This clearly indicates that the samples prepared with this new matrix are well suited for high-throughput DNA analysis.

Analysis of mixtures

The use of DABP as a matrix to analyze mixtures of oligonucleotides was the final study of this present work. This is important because genotyping requires analysis of multiple oligonucleotides. Figure 8a shows a typical spectrum of a mixture of 12 different oligonucleotides prepared at

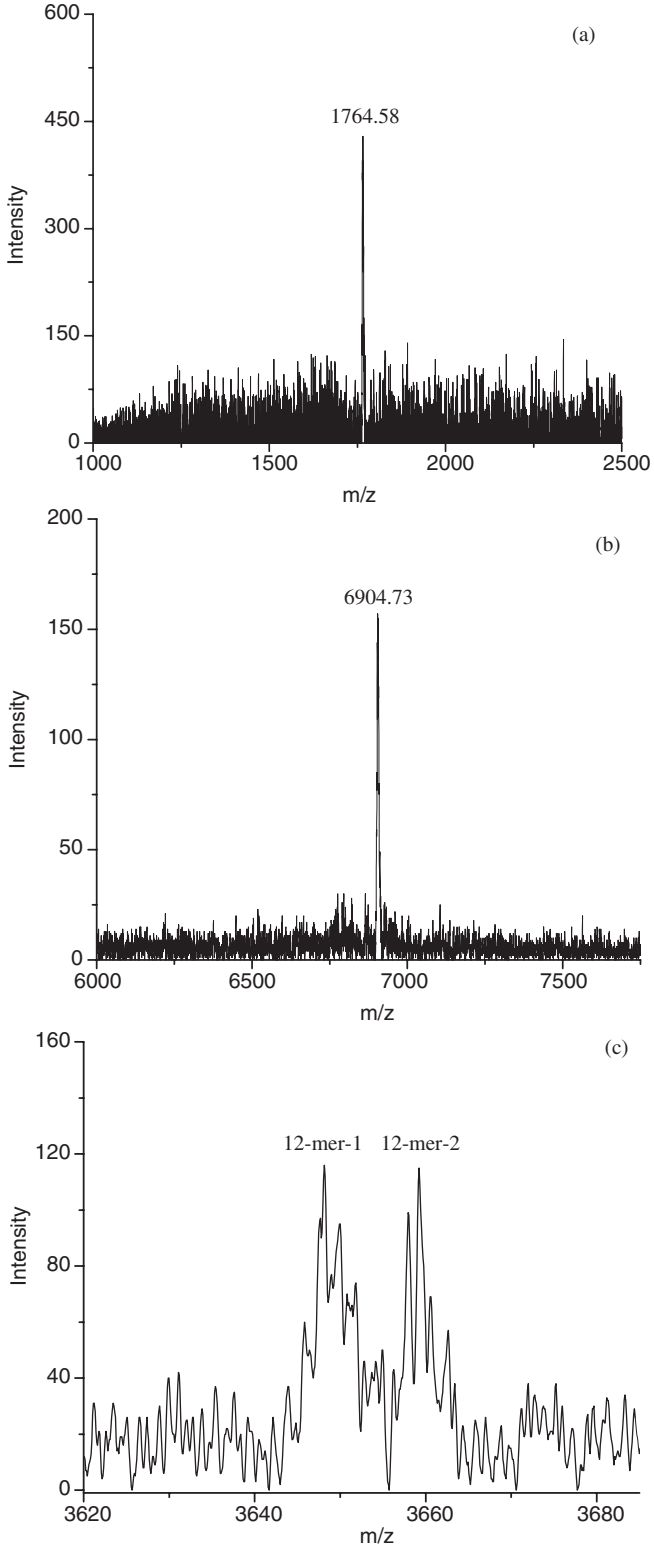

Figure 6. MALDI mass spectra of (**a**) 70 amol of d(T)6; (**b**) 62.5 fmol of 23mer-1; and (**c**) 12.5 fmol of 12mer-1 and 12mer-2.

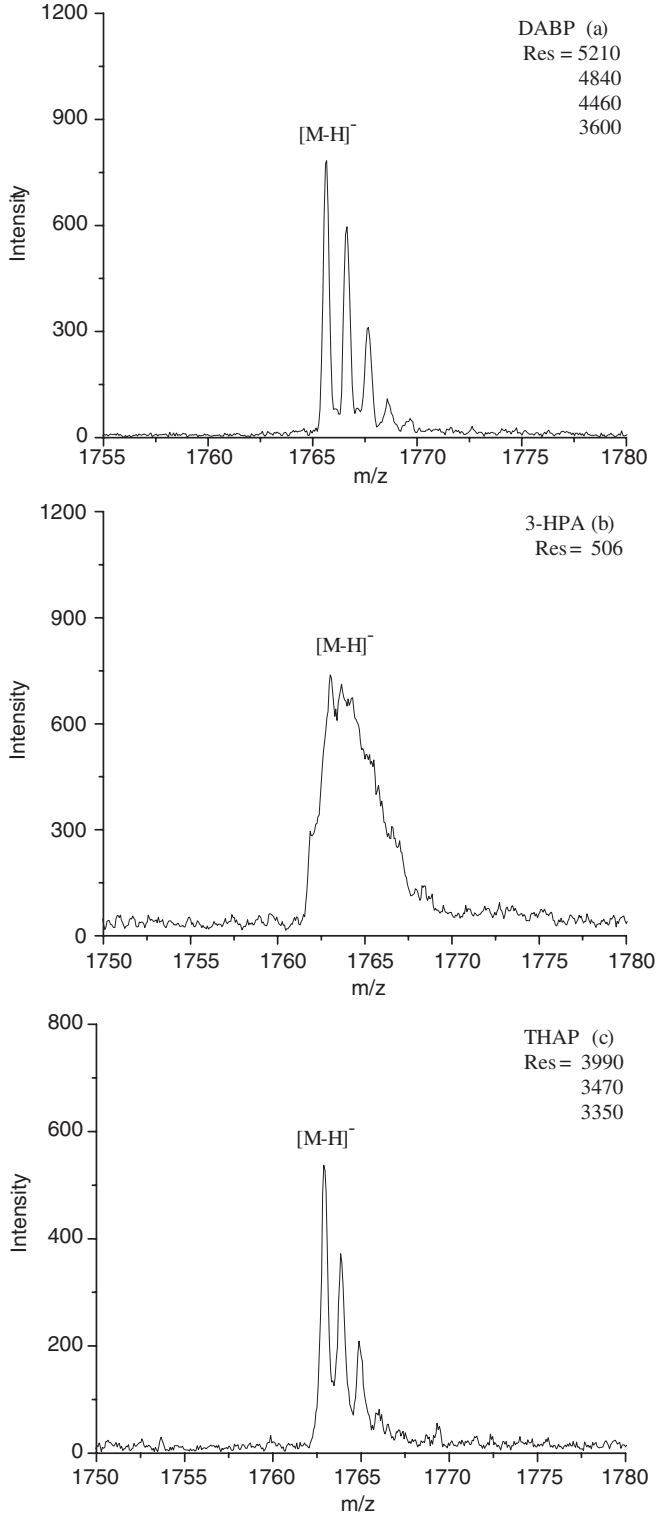

Figure 7. MALDI mass spectra of $d(T)_6$ obtained with matrices of (**a**) DABP, (**b**) 3-HPA and (**c**) 2,4,6-THAP. The quantity of the oligonucleotide loaded was 1.4 pmol.

equivalent molar concentrations. All 12 oligonucleotides were 18mer and their sequences along with the mass difference between the neighboring peaks are listed in Table 4. As seen from Table 4, the 12 oligos segregate into 8 molecular weights (4 oligos have the same molecular weight as other oligo molecules). Figure 8b displays the expanded portion of Figure 8a. The labeled numbers in Figure 8b correspond to the oligos indicated in Table 4. It

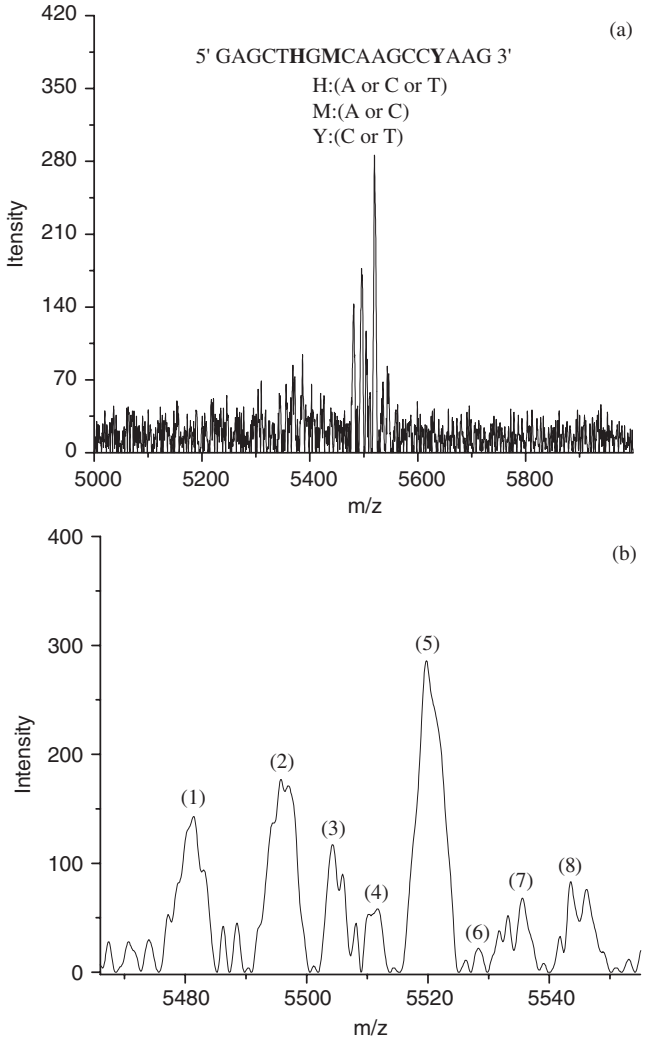

Figure 8. MALDI mass spectra of (**a**) 5 pmol of a mixture of 12 different oligonucleotides; (**b**) the expanded spectrum (a) in the mass region from 5465 to 5555 Da.

Table 4. Sequences, molecular weights and mass differences of 12 oligonucleotides

N	Sequences	Differences $\Delta = \text{Mass}_n - \text{Mass}_{n-1}$ (Da)
①	5′-GAGCT**C**G**C**CAAGCC**C**AAG-3′	
②	5′-GAGCT**T**G**C**CAAGCC**C**AAG-3′	15
	5′-GAGCT**C**G**C**CAAGCC**T**AAG-3′	
③	5′-GAGCT**C**G**A**CAAGCC**C**AAG-3′	9
	5′-GAGCT**A**G**C**CAAGCC**C**AAG-3′	
④	5′-GAGCT**T**G**C**CAAGCC**T**AAG-3′	6
⑤	5′-GAGCT**T**G**A**CAAGCC**C**AAG-3′	9
	5′-GAGCT**C**G**A**CAAGCC**T**AAG-3′	
	5′-GAGCT**A**G**C**CAAGCC**T**AAG-3′	
⑥	5′-GAGCT**A**G**A**CAAGCC**C**AAG-3′	9
⑦	5′-GAGCT**T**G**A**CAAGCC**T**AAG-3′	6
⑧	5′-GAGCT**A**G**A**CAAGCC**T**AAG-3′	9

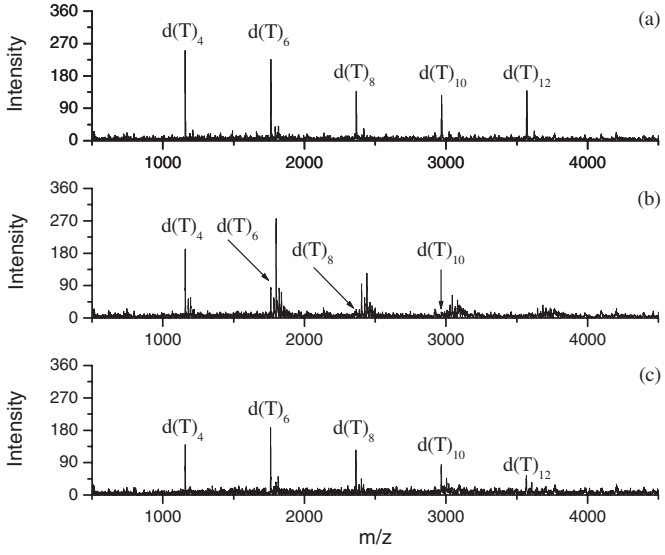

Figure 9. MALDI mass spectra of an oligonucleotide mixture obtained with matrices of (**a**) DABP, (**b**) 2,4,6-THAP and (**c**) 3-HPA, respectively. The quantity of each oligonucleotide loaded was 500 fmol.

is seen that this mixture of oligonucleotides were well resolved with eight ion peaks located at their respective expected mass positions. This result further demonstrates the excellent resolving power of this new matrix, as evident by the fact that two oligos differing by only 6 Da can be clearly resolved as indicated in Figure 8b. This is significant because the difficulty in resolving A from T (9 Da difference) has been a problem often encountered in the MALDI-TOF-based genotyping approaches. However, whether peak 6 is the shoulder on the signal from oligonucleotide7 still needs further investigation.

Figure 9 shows the mass spectra of $d(T)_{4,6,8,10,12}$ obtained using matrices of DABP, 2,4,6-THAP and 3-HPA, respectively, with 500 fmol of each oligonucleotide loaded. As shown in Figure 9, this mixture of oligonucleotides can also be well resolved with no fragmentation and fewer adducts with DABP as a matrix while alkali metal cation adducts dominate the mass spectrum with 2,4,6-THAP. Although the mixture can be resolved by 3-HPA, the quality of the mass spectrum is not as good as that obtained with DABP. In addition, more effort was required to find good shots in obtaining the spectrum shown in Figure 9b.

CONCLUSION

A new matrix of DABP has been evaluated for the analysis of oligonucleotides by MALDI-TOF. DABP was found to be a superior matrix for the detection of intact oligonucleotides smaller than 30mer without the use of any other additives or co-matrices. The use of this new matrix in MALDI can greatly improve the MALDI performance with respect to salts tolerance, reduction of oligonucleotide fragmentation and significant improvement of the detection sensitivity and mass resolution, compared with conventional matrices. The facile sample preparation and the reduced laser powers required for analysis add to the advantages of using DABP as a matrix. In addition, the DABP prepared samples are much more homogenous, reducing the need for finding the 'sweet spots'. Therefore, DABP has a great potential for

use in the MALDI-MS analysis of complex, and or low-concentration DNA samples.

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Conflict of interest statement. None declared.

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