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Reducing the Alkali Cation Adductions of Oligonucleotides Using Sol−Gel-Assisted Laser Desorption/Ionization Mass Spectrometry

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The alkali cation adductions of oligonucleotides dramatically degrade MALDI mass spectra and even affect the detection limit. Desalting is generally involved in MALDI sample preparation. This work demonstrates the feasibility of using 3,4-diaminobenzoic acid (DABA) and 3,5-DABA as the MALDI matrix for oligonucleotide analysis. Furthermore, sodium ion adducts of oligonucleotides were simultaneously reduced in the mass spectra when DABA was used as the MALDI matrix and sol−gel material was used as the sample support. However, depositing the sample on the sample support was very difficult, and the lack of homogeneity of analytes/matrix distribution on the sample support also led the analyte signals to be revealed only in "sweet spots". Alternatively, DABA was doped into sol−gel materials to generate homogeneous DABA/sol−gel hybrid film. The DABA/sol−gel hybrid film was used as the sample substrate to assist the desorption/ionization of analytes. The analyte signals were evenly found on the sample substrate. The sodium ion adductions of oligonucleotides were also effectively suppressed. The sample preparation used in this approach resembles that used in the authors' previous study, involving sol−gel-assisted laser desorption/ionization (SGALDI) mass spectrometry (Lin, Y.-S.; Chen, Y.-C. ***Anal. Chem.*** **2002,** ***74,*** **5793−5798.) The SGALDI approach was demonstrated to be effective in assisting the desorption/ionization of peptides and small proteins. Herein, the SGALDI material, DABA/sol−gel hybrid material, was successfully applied to oligonucleotide analysis, and good-quality mass spectra were obtained without extra desalting. Additionally, the presence of 0.1% SDS in the oligonucleotide sample solution was tolerated without degrading the mass spectra. The largest detectable molecular size for oligonucleotides was 72 mer. The detection limit for 24 mer of oligonucleotide was 20 fmol.**

Phosphodiester backbones in oligonucleotides have a high affinity for alkali cations. In particular, since sodium ions are ubiquitous in the metal target using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, sodiated oligonucleotide ions generally dominate MALDI mass spectra. Reducing cation adductions of oligonucleotides can improve the quality of mass spectra and the detection limits of oligonucleotides in MALDI analysis. Although using cation-exchange resin beads can remove most cations from oligonucleotide solutions,[1−3] a more effective and straightforward approach is to add comatrix to the sample preparation of MALDI for oligonucleotide analysis.[3−13] Comatrixes, such as ammonium salts, were first used to inhibit the alkali cation adductions of oligonucleotides.[6−8] Neutral amines, such as triethylamine[4,5] and tetraamine spremine,[9,10] have been proven to be as effective matrix additives for inhibiting the alkali cation adductions of oligonucleotides. In approaches that use such amines, the matrix additives are presumably involved in replacing alkali cations with amino functional groups in MALDI analysis. The matrixes commonly used for oligonucleotide analysis are 3-hydroxypicolinic acid (3-HPA), 6-aza-2-thiothymine and 2,4,6-trihydroxyacetophenone.[3−13] However, a matrix like 3-HPA is incompatible with amine comatrixes.[12] 3-HPA normally combines with diamino citrate as the MALDI matrix for oligonucleotide analysis in MALDI. Acidified HPA was also used in the positive ion mode to analyze oligonucleotides with high masses, providing good mass resolution and mass accuracy.[13] The selections of matrixes and comatrixes determine the quality of MALDI mass spectra of oligonucleotides. If a molecule has sufficient absorption capacity to be used as a MALDI matrix and also includes several amino groups in a single molecule, it can be potentially used as both the MALDI matrix and the desalting reagent, simultaneously.

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Thus, concern about the compatibility between the matrix and the comatrix does not arise in the sample preparation. Additionally, the homogeneity of the matrix/sample coverage on the sample target is also essential for obtaining shot-to-shot reproducibility. Polymeric materials have been proposed for use as the sample support in generating homogeneous samples,[13−21] and alkali cation adductions were also effectively inhibited by the use of hydrophobic polymers. The sensitivity and tolerance to salt have thus been improved.[13−21]

In this study, diaminobenzoic acid (DABA), including 3,4-DABA and 3,5-DABA, was discovered to be applicable as a UV-MALDI matrix, owing to its good molar absorptivity at wavelength of 337 nm. Two amino functional groups attached to the aromatic ring in DABA can also suppress the cation adductions of oligonucleotides during analysis. The metal target generally unavoidably contains a large number of alkali ions, including sodium and potassium ions. Thus, the suppression effect by reducing sodium ion adductions was observed only when a mixture of analyte/aminobenzoic acid was loaded on a hydrophobic sample support, such as Parafilm membrane. However, this approach did not effectively suppress sodium ion adductions of oligonucleotides larger than 12 mer. Thus, an alternative approach was taken to improve the results.

The authors recently developed a new type of laser desorption mass spectrometry, sol−gel-assisted laser desorption/ionization (SGALDI) mass spectrometry,[22] which uses sol−gel derivate as the sample substrate to assist the desorption/ionization of analytes. The sol−gel derivative was prepared by entrapping a conventional MALDI matrix, 2,5-dihydroxybenzoic acid (2,5-DHB), in the sol−gel structure. The sol−gel-derived 2,5-DHB film was proven to be an effective material for assisting the desorption/ionization of analytes, including peptides and small proteins. Furthermore, the SGALDI mass spectra showed the protonated pseudomolecular ions of analytes dominated the mass spectra and no alkali cation adducts were observed. The results implied that this approach to oligonucleotide analysis might yield low intensities of sodiated analyte ions. However, when sol−gel-derived 2,5-DHB was used as the sample substrate and applied to oligonucleotide analysis, the sodium ion adductions of oligonucleotides were not suppressed during SGALDI analysis. One of the unique features of the sol−gel technique is that any organics with functional groups such as hydroxyl or amine groups can be easily doped into the sol−gel structure.[23,24] On the basis of previous studies on using amine or amino-containing comatrixes to reduce the alkali cation adductions of oligonucleotides,[3−12] we proposed a sol−gel hybrid material by entrapping molecules with amino functional groups and good molar absorptivity at 337 nm into the sol−gel net structure. The newly entrapped molecules were found originally by replacing the two hydroxyl groups in DHB with two amino groups. DABAs, therefore, became perfect candidates for use as dopants in the SGALDI sample substrate. As expected, the alkali cation adductions reduced drastically when the DABA/sol−gel hybrid material was used as the sample substrate in SGALDI for oligonucleotide analysis. Both 3,4- DABA/sol−gel and 3,5-DABA/sol−gel hybrid materials were demonstrated to be effective sample substrates in SGALDI analysis. Homogeneous sample deposition on the sample substrate was achieved using this approach. Synthesized oligonucleotides were used as the samples throughout this study.

EXPERIMENTAL SECTION

Reagents. Tetraethoxysilane (TEOS, MW = 208.33, density 0.934) was purchased from Fluka (Buchs, Switzerland). 3,4-Diaminobenzoic acid and emeraldine (MW_{av} = 10 000) were purchased from Aldrich (Milwaukee, WI). Bovine serum insulin was obtained from Sigma (MO). Methanol was obtained from Pharmca (Brookfield, CT). All the synthesized oligonucleotides were purchased from Biobasic Inc. Hydrochloric acid was purchased from Merck (Darmstadt, Germany). All the reagents were used as received.

Sample Preparation on Hydrophobic Films. DABA matrix solution (15 mg/mL) was prepared in acidified methanol (methanol:HCl (30%) = 80:3 (v/v)). The sample solution was prepared by mixing equal volumes of DABA solution with oligonucleotide solution. A 0.2-μL aliquot of the sample solution was subsequently applied to Parafilm, which had previously been attached to a sample target using double-sided carbon tape (Ted Pella). After the volatile solvents had evaporated, the target was put in the mass spectrometer for analysis.

The pure sol−gel solution was prepared by mixing 4.5 mL of TEOS, 1 mL of water, and 0.4 mL of hydrochloric acid (0.01 N). The mixture was stirred for 3 h. Generally, TEOS took 3 h to achieve complete hydrolysis. A 0.2-μL sample of this sol−gel solution was deposited on Parafilm, which had been previously attached to a sample target using double-sided carbon tape. After a film formed (which took ∼30 min), 0.3 μL of the mixture, containing equal volumes of the oligonucleotide solution and DABA solution (1:1, v/v), was deposited on the film. The sample target was loaded into the mass spectrometer after volatile solvents in the sample had evaporated.

SGALDI Sample Preparation. The 3,4-DABA/sol−gel hybrid material was prepared by mixing 0.1134 g of 3,4-DABA, 1.6 mL of methanol, 60 μL of hydrochloric acid (30%), 1.4 mL of water, and 4.5 mL of TEOS. The 3,4-DABA/sol−gel mixture was stirred at room temperature (26 ± 2 °C) for 24 h before use. The 3,5-DABA/sol−gel hybrid material was prepared by mixing 0.1327 g of 3,5-DABA, 1.6 mL of methanol, 60 μL of hydrochloric acid (30%), 1.4 mL of water, and 4.5 mL of TEOS. The 3,5-DABA/sol−gel mixture was stirred at room temperature for 24 h before use. A 0.25-μL sample of the DABA/sol−gel hybrid material was then applied to a Parafilm membrane, which had already been attached to a sample plate using double-sided black carbon tape. The sol−gel product was very difficult to wash off the surface of the sample

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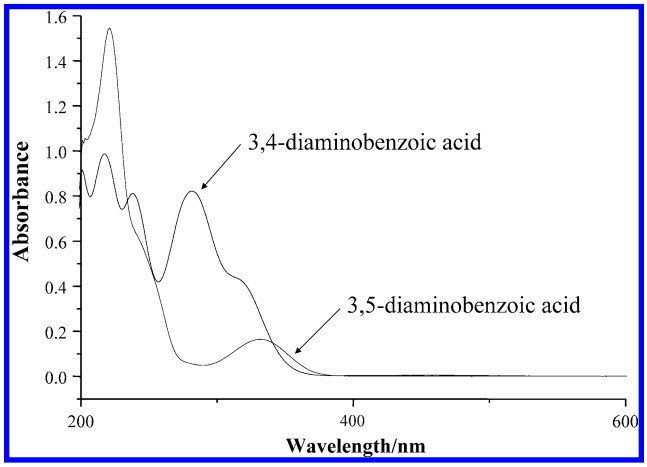

Figure 1. UV spectra of 3,4-DABA and 3,5-DABA dissolved in methanol.

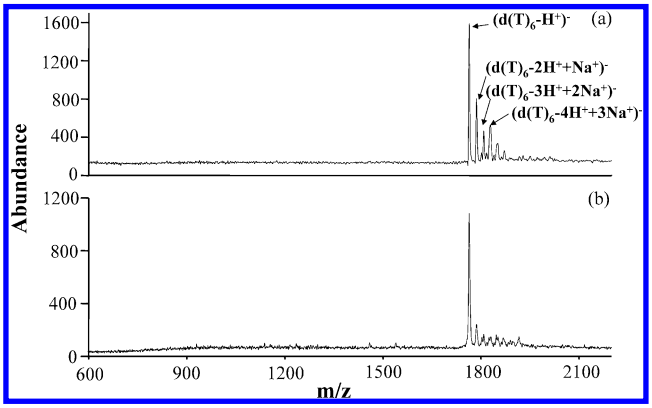

Figure 2. (a) MALDI mass spectrum of $d(T)_6$ (5.67 pmol), obtained by depositing the mixture of analyte with 3,4-DABA directly on the metal target. (b) MALDI mass spectrum of $d(T)_6$ (5.67 pmol), obtained by depositing the mixture of analyte with 3,4-DABA on a Parafilm membrane.

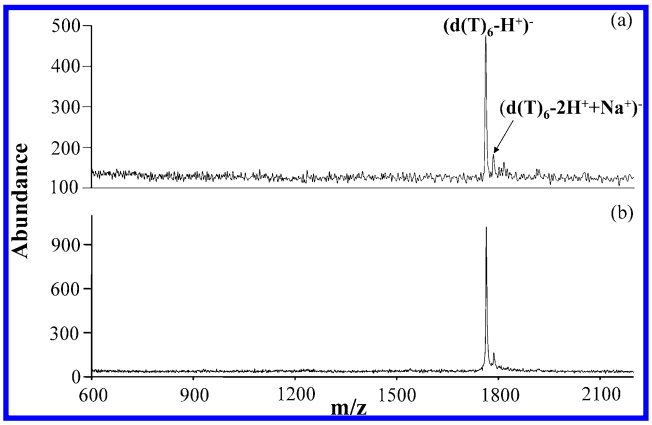

Figure 3. (a) MALDI mass spectrum of $d(T)_6$ (85 pmol), obtained by depositing the mixture of analyte with 3,4-DABA on a sol−gel film. (b) SGALDI mass spectrum of $d(T)_6$ (85 pmol) using 3,4-DABA/sol−gel hybrid film as the sample substrate.

plate after it had been directly deposited on it. The Parafilm was used as the support for the sol−gel film on the sample plate to prevent this problem. After a thin film had formed (taking ∼30 min) on the Parafilm membrane, the sample solution (0.15 μL) was deposited on top of it. The sample solution homogeneously spread on the sample substrate. The sample was ready for mass spectral analysis after the volatile solvent had evaporated (which took ∼20 min).

Instruments. All the mass spectra were obtained in negative ion mode using a Biflex III (Bruker) linear time-of-flight mass spectrometer. The mass spectrometer was equipped with a 337-nm nitrogen laser, a 1.25-m flight, and a sample target with the capacity to load 384 samples simultaneously. The accelerating voltage was set to 19 kV. Inductively coupled plasma mass spectrometry (ICPMS, Agilent 7500S, Japan) was used to determinate the salt content for oligonucleotide samples. The oligonucleotide sample (1 mg/mL) was diluted 10^4 times by ultrapure water before ICPMS analysis.

RESULTS AND DISCUSSION

The UV spectra of DABA dissolved in methanol show that 3,5-DABA has an absorption coefficient of 2400 cm^{-1} M^{-1} at wavelength of 337 nm, while 3,4-DABA has an even better absorption capacity, with an absorption coefficient of 2995 cm^{-1} M^{-1} at the same wavelength (Figure 1). These UV spectral results imply that both DABAs can be used as UV-MALDI matrixes.

Figure 2a is the MALDI mass spectrum of $d(T)_6$ (5.67 pmol), obtained by depositing an equal volume (0.1 μL) of the analyte (0.1 mg/mL) with 3,4-DABA (15 mg/mL) directly on a metal target for MALDI analysis. The mass spectrum reveals a series of sodium adduct ions of $d(T)_6$. Figure 2b is the MALDI mass spectrum of $d(T)_6$ (5.67 pmol), obtained by depositing the same sample as that used in Figure 2a on Parafilm. The sodium ion adducts of $d(T)_6$ are slightly reduced. Although the results are consistent with those in earlier studies, which found that a hydrophobic polymer support can improve the cation tolerance, this effect is limited by the sizes and concentrations of oligonucleotides.[25] When the amount of $d(T)_6$ was increased by 1 order (56.7 pmol) for analysis, the sodiated oligonucleotide pseudomolecular ions became notable (results not shown). A series of sodium ion adducts of $d(T)_6$ appeared in the MALDI mass spectrum. Higher concentrations of oligonucleotides require a higher salt tolerance. Furthermore, the suppression effects were only observed in the MALDI mass spectra of oligonucleotides smaller than 12 mer.

A hydrophobic sol−gel film was used as an alternative sample support, which was proposed to be capable of excluding some of the sodium ions during analysis. When an equal volume (0.15 μL) of $d(T)_6$ (1 mg/mL) with 3,4-DABA (15 mg/mL) was directly deposited on a pure sol−gel film, the deprotonated pseudomolecular ion of $d(T)_6$ dominated the mass spectrum (Figure 3a). The sodiated pseudomolecular ions of $d(T)_6$ are effectively suppressed in the mass spectrum. The concentration of sodium ions in this $d(T)_6$ sample was ∼258 ppm, which was determined by ICPMS. However, directly applying the sample solution on the pure sol−gel film was difficult because of the large polarity differences between the sample solution and the film. Thus, the sample/matrix could not homogeneously spread on the sol−gel film, and some effort was made to find the sweet spots in which analyte signals could be obtained. To solve this problem about the inhomogeneity of sample/matrix on the sol−gel film, a homogeneous sol−gel film was prepared by premixing the DABA with the sol−gel material, making the film more hydrophilic

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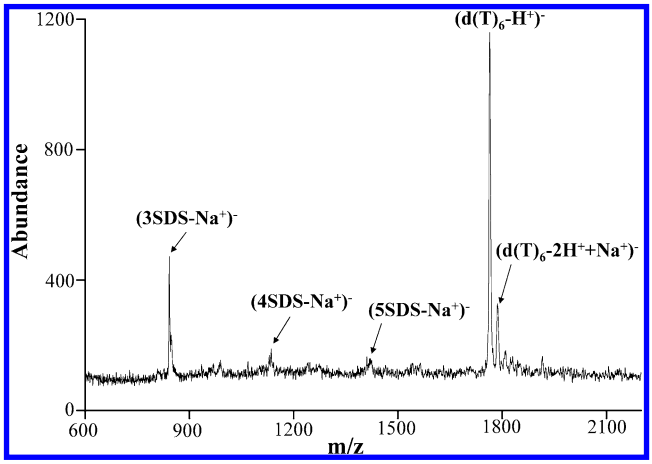

Figure 4. SGALDI mass spectrum of $d(T)_6$ (425 fmol), obtained with the addition of 0.1% SDS in the sample solution using 3,4-DABA/sol−gel hybrid material as the sample substrate.

because of the presence of DABA. The DABA/sol−gel hybrid material was then used as the sample substrate in assisting the desorption/ionization of analytes. Figure 3b shows the SGALDI mass spectrum of $d(T)_6$ (85 pmol), obtained using sol−gel DABA hybrid material as the sample substrate. The deprotonated pseudomolecular ions of $d(T)_6$ dominated the mass spectrum. The spectrum exhibits only a weakly sodiated oligonucleotide pseudomolecular ion peak next to the deprotonated pseudomolecular ion peak of $d(T)_6$. The analyte signals could be homogeneously observed in the sol−gel film. The result successfully demonstrates that the sol−gel/DABA hybrid material can effectively assist desorption/ionization, while simultaneously inhibiting the sodium ion adducts of oligonucleotides. Similar results were obtained in SGALDI analysis using the sol−gel/3,5-DABA hybrid material as the sample substrate. The amount of $d(T)_6$ used for analysis herein was much higher than that in practical use in conventional MALDI analysis. The experiment intended to demonstrate that this SGALDI approach could have high salt tolerance since higher concentrations of oligonucleotides employed for MALDI analysis usually require a high salt tolerance.[25]

To further evaluate the salt tolerance of this SGALDI approach, extra salt was added into oligonucleotide samples for examination. Figure 4 shows the SGALDI mass spectrum of $d(T)_6$ (425 fmol), obtained with the addition of 0.1% sodium dodecyl sulfate (SDS) in the sample solution (w/v). The sample substrate was 3,4-DABA/sol−gel hybrid material. The deprotonated pseudomolecular ion peak of $d(T)_6$ dominates the mass spectrum, and a weak peak of the sodiated peak is adjacent to it. The presence of 0.1% SDS in the oligonucleotide sample solution does not degrade the mass spectrum. The desodiated trimer $((3SDS - Na^+)^-)$, tetrameter $((4SDS - Na^+)^-)$, and pentamer ions $((5SDS - Na^+)^-)$ of SDS at m/z 841, 1129, and 1417, respectively, are observed in the same mass spectrum. The result indicates this approach can tolerate 1000 ppm of extra salt in the sample solutions.

Using DABA/sol−gel hybrid material as the sample substrate in SGALDI analysis cannot only reduce the sodiated ions for polythymidylic acid, but it can also effectively suppress the sodium ion adductions of mixed-base oligonucleotides. Figure 5 shows the SGALDI mass spectrum of a mixed-base oligonucleotide, i.e., d(CCTCTGGTCTCC) (423 fmol). The sodiated oligonucleotide ions are effectively suppressed. The deprotonated pseudomolecular ion $(M - H^+)^-$ at m/z 3547 dominates the mass spectrum, while the doubly charged molecular ion $(M - 2H^+)^{2-}$ appears at m/z 1773. The McLuckey fragmentation nomenclature system[26] was employed to denote the fragments. Additionally, the peaks revealed at m/z 2456, 2376, 1904, 1822, 1742, 1575, 1493, and 1413 correspond to the w_8, y_8, a_7-B_7, w_6, y_6, a_6-B_6, w_5, and y_5 fragments of the oligonucleotide, respectively. Although the intensities of some fragments in Figure 5 were quite low, the m/z values of these fragments were confirmed when the sample amount was increased 10 times (results not shown). The fragmentation pathway presumably was initiated by a base loss (B_4, B_6, and B_7) followed by backbone fragmentation, which had been suggested by many studies.[27−30] Guanine and cytosine have higher proton affinity than that of thymine,[28] so the initial fragmentation step presumably was started from a guanine and a cytosine loss. That two weak peaks revealed at m/z 3398 and 3437 correspond to the fragments of $(M - H^+ - B_{6(7)})^-$ and $(M - H^+ - B_4)^-$ by losing a guanine (G) and a cytosine (C) from the intact molecule, respectively, implies this fragmentation pathway.

Figure 6a shows the SGALDI mass spectrum of $d(T)_{36}$ (138 fmol). In addition to the pseudomolecular ion $((dT)_{36} - H^+)^-$ and the doubly charged ion $((d(T)_{36} - 2H^+)^{2-})$, a series of y_n fragment ions with 304 amu difference at m/z 3587, 3981, 4195, ... appear in the low-mass region. Based on mass calculation, a very weak peak with an 80 amu difference adjacent to y_n is w_n fragment denoted by an asterisk in Figure 6a. The w_n series ions are barely observed in the mass spectrum due to low mass resolution and intensity. Figure 6b is the SGALDI mass spectrum of $d(T)_{24}$ (20 fmol). The doubly charged ion $(d(T)_{24} - 2H^+)^{2-}$ and some weak fragment ions were observed in the low-mass region. This result also indicates that the detection limit of the SGALDI approach for 24 mer of oligonucleotides is lower than 20 fmol.

How the desalting occurs in this SGALDI approach is of interest. The mechanism is presumably similar to that of using tetraamine spermine and ammonium ions as desalting agents.[4−10] Protonated DABA binds to the phosphate backbone of oligonucleotides and releases protons to the phosphate groups. According to the experiments performed in this work, when TEOS was hydrolyzed in a basic environment, the DABA/sol−gel hybrid film did not suppress the sodium ion adductions of oligonucleotides in SGALDI analysis. Furthermore, when oligonucleotides were analyzed using unacidified DABA as the MALDI matrix, the sodium ion adductions of oligonucleotides were not suppressed. The lack of a source of protons for DABA molecules may result in failure of releasing protons from DABA to the oligonucleotides during analysis. The presence of many protons is crucial for the desalting process. Polyalanine has a good absorption capacity (absorption coefficient $\epsilon_{337} = 5.36 \times 10^5\ cm^{-1}\ M^{-1}$) at a wavelength of 337 nm based on UV absorption spectroscopic analysis and

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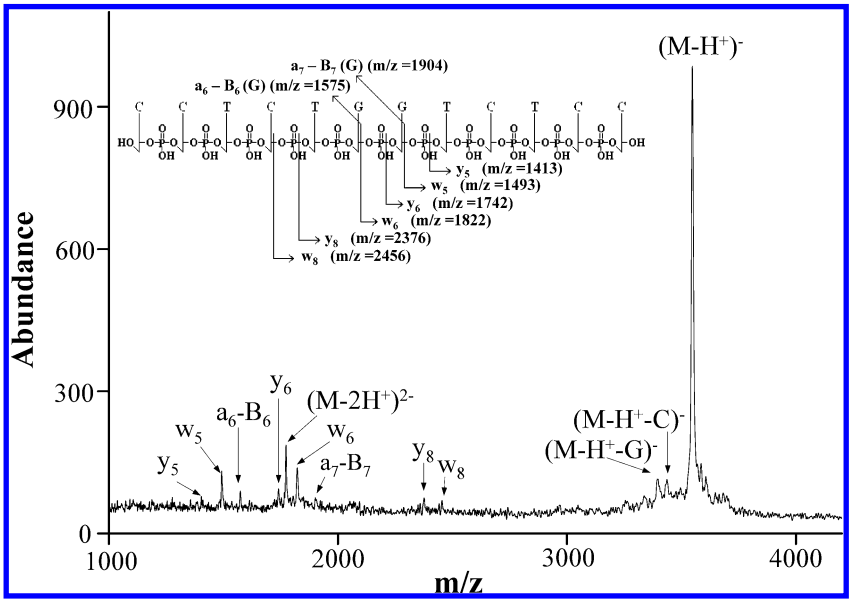

Figure 5. SGALDI mass spectrum of d(CCTCTGGTCTCC) (423 fmol) using 3,4-DABA/sol−gel hybrid film as the sample substrate.

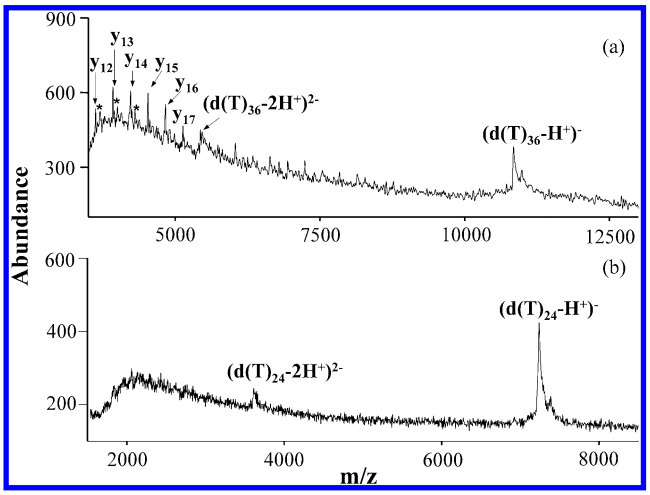

Figure 6. SGALDI mass spectra of (a) $d(T)_{36}$ (138 fmol) and (b) $d(T)_{24}$ (20 fmol) using 3,4-DABA/sol−gel hybrid material as the sample substrate. The w_n series ions are marked with an asterisk in (a).

also consists of several secondary aromatic amines in the structure. If protonated polyalanine can bind to the phosphate backbone of oligonucleotides and release protons to the phosphate, the desalting process may occur. Polyalanine, i.e., emeraldine (MW_{av} = 10 000), sol−gel hybrid material was used as the sample substrate for SGALDI analysis. However, only proteins and peptides were observed in the SGALDI mass spectra obtained using polyalanine/sol−gel hybrid material as the SGALDI sample substrate (results not shown.) Obtaining any DNA signals was difficult. Polyalanine/sol−gel hybrid material failed to play the role as a desalting agent as the DABA/sol−gel hybrid material does in SGALDI analysis for oligonucleotides. Presumably, the polyalanine was too big to approach the phosphate backbone of oligonucleotides and therefore is limited in its effectiveness in desalting.

CONCLUSIONS

DABA/sol−gel hybrid materials have been demonstrated to be effective sample substrates and desalting materials for oligonucleotide analysis in SGALDI analysis. Sample preparation in SGALDI analysis does not require the sample solution to be premixed with the matrix. Thus, this SGALDI approach does not raise issues about the cocrystallization of the matrix with the analytes and the compatibility of the matrix with the comatrix. Additionally, good shot-to-shot reproducibility and homogeneous sample deposition on the DABA/sol−gel hybrid film are achieved. The quality of the mass spectra is rather good, leading to a low detection limit and a fair mass range. A 72 mer oligonucleotide is the largest nucleotide yet detected by the SGALDI approach. Nevertheless, bovine serum albumin with a molecular weight of ~66 000 was detected using this approach (results not shown). Accordingly, the mass range can be extended to larger oligonucleotides by improving the sample preparation. Other suitable sizes of molecules that contain more than two amino groups with good molar absorptivities at a wavelength of 337 nm may be used as SGALDI dopants to extend further the mass range of oligonucleotides in SGALDI analysis.

The current approach requires more time in sample preparation than that of the conventional MALDI approach. However, the time-consuming procedures may be simplified by modifying the sample preparation procedures. Owing to the nature of sol−gel material, covalent bonds are formed between sol−gel derivatives and the surface of glass or glasslike materials after spin-coating sol−gel solution on the glass surface. Thus, the preparation time can be potentially reduced by alternatively using glass chips coated with DABA/sol−gel hybrid material as the SGALDI sample substrate. A number of glass chips coated with DABA/sol−gel hybrid material can be made beforehand. Thus, it is unnecessary to prepare the DABA/sol−gel hybrid material every time the SGALDI analysis is performed. Elimination of the preparation of the DABA/sol−gel hybrid material may greatly reduce the preparation time for SGALDI analysis. That is, sample preparation only requires the step of applying sample solution on the glass chip if the glass chip has been made in advance. It is then ready to send the glass chip into the mass spectrometer for analysis after the volatile solvents in sample have evaporated. Examination

of the effectiveness using a modified glass chip as the sample substrate for SGALDI analysis is in progress in our laboratory.

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