

Using sol–gel/crown ether hybrid materials as desalting substrates for matrix-assisted laser desorption/ionization analysis of oligonucleotides

Mao-Feng Weng and Yu-Chie Chen*

Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan

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This study demonstrates the feasibility of using sol–gel/crown ether hybrid materials as sample substrates that reduce the intensity of the signals of sodium ion adducts of oligonucleotides during matrix-assisted laser desorption/ionization (MALDI) analysis. 2-Hydroxymethyl[15]crown-5 and 2-hydroxymethyl[18]crown-6 were added as dopants during the sol–gel process to generate desalting substrates for MALDI sample deposition. The results demonstrate that the sol–gel/crown ether hybrid materials effectively suppress the formation of sodiated oligonucleotides during MALDI analysis. The largest detectable molecular size for an oligonucleotide was a 100-mer, and the detection limit for an oligonucleotide 36-mer was ca. 20 fmol. Copyright © 2004 John Wiley & Sons, Ltd.

Generally, there are two problems that arise during matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) analysis of oligonucleotides. The first is that serious levels of fragmentation are frequently observed to occur from oligonucleotides having sequence lengths larger than 12 bases. Secondly, the presence of alkali cation adducts of oligonucleotides always results in poor mass spectral quality and high detection limits. Thus, an ongoing goal in MALDI analysis of oligonucleotides is reduction of cation adducts to improve the quality of mass spectra and to lower the detection limits. For example, use of cation-exchange resin beads to remove cations from oligonucleotide solutions,^{1–3} and addition of a co-matrix during sample preparation,^{3–13} have been applied successfully to DNA analysis using MALDI. Co-matrices, such as ammonium salts,^{6–8} triethylamine,^{4,5} and the tetraamine spermine,^{9,10} have proven to be effective matrix additives for inhibiting the formation of alkali cation adducts. These matrix additives are presumably involved in replacing alkali cations by amino functional groups during MALDI analysis.

The matrices commonly used for oligonucleotide analysis are 3-hydroxypicolinic acid (3-HPA), 6-aza-2-thiothymine (6-ATT), and 2,4,6-trihydroxyacetophenone (2,4,6-THAP).^{3–15} The selection of the matrix and co-matrix is the main determinant of the quality of MALDI mass spectra of oligonucleotides.

In a previous study¹⁶ we demonstrated that the intensities of the signals of alkali cation adducts are reduced drastically when diaminobenzoic acid (DABA)/sol–gel hybrid materials are used as the sample substrate for MALDI analysis of oligonucleotides. DABA/sol–gel hybrid materials are

suitable MALDI matrices because of their good absorption coefficients at a wavelength of 337 nm. Homogeneous sample deposition on the sol–gel/DABA hybrid sample substrate is achieved. The sol–gel/DABA hybrid material functions as both a desalting agent and a matrix for the MALDI analysis. The desalting processes presumably involve binding of protonated DABA to the phosphate backbone of the oligonucleotide, thus releasing protons to the phosphate groups.

As an alternative strategy, crown ethers are likely candidates for the role of desalting agents in the MALDI analysis of oligonucleotides. Crown ethers are macrocyclic polyethers capable of forming stable complexes with alkali and alkaline earth metal ions; e.g., [15]crown-5 and [18]crown-6 readily form complexes with alkali ions.^{17,18} Based on these characteristics, we chose to test crown ethers as desalting agents to sequester sodium ions from oligonucleotide samples during MALDI analysis. To demonstrate the feasibility of using crown ethers in this way, we doped crown ethers into sol–gels to generate homogeneous substrates for MALDI sample deposition, and then deposited sample solutions containing both matrix and oligonucleotides onto the sol–gel substrate. Our results demonstrate that use of sol–gel/crown ether hybrid materials as sample substrates is an effective method for reducing the extent of sodium ion adduction of oligonucleotides during MALDI analysis.

EXPERIMENTAL

Reagents

Tetraethoxysilane (TEOS) and 2-hydroxymethyl[18]crown-6 were purchased from Fluka (Buchs, Switzerland); methanol was from Pharmacia (Brookfield, CT, USA), and hydrochloric acid from Merck (Darmstadt, Germany). 2-Hydroxymethyl[15]crown-5, 3-hydroxypicolinic acid (3-HPA), 6-aza-2-thiothymine (6-ATT), and sinapinic acid (SA) were obtained from Aldrich (Milwaukee, WI, USA), sodium

*Correspondence to: Y.-C. Chen, Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan.

E-mail: yuchie@mail.nctu.edu.tw

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chloride from Riedel de Hën (Deisenhofen, Germany), and dimethylformamide (DMF) from Tedia (OH, USA). All the synthesized oligonucleotides were purchased from Biobasic Inc. (Canada). All reagents were used as received.

Preparation of sol-gel/crown ether hybrid materials

A mixture of 2-hydroxymethyl[15]crown-5 (0.1, 0.2, or 0.3 M) doped in a sol-gel was prepared by mixing 2-hydroxymethyl[15]crown-5 (2.6, 5.3, or 7.9 mg) with hydrochloric acid (0.01 N, 6.8 μ L), water (17 μ L), and TEOS (76 μ L). The mixture was stirred at room temperature ($26 \pm 2^\circ\text{C}$) for 4 h before use. A 0.3 M 2-hydroxymethyl[18]crown-6/sol-gel hybrid material was prepared by mixing 2-hydroxymethyl[18]crown-6 (9.1 mg) with hydrochloric acid (0.01 N, 6.8 μ L), water (17 μ L), and TEOS (76 μ L). The mixture was stirred at room temperature ($26 \pm 2^\circ\text{C}$) for 4 h before use.

Sample preparation for MALDI analysis

The sol-gel/crown ether hybrid material (0.5 μ L) was deposited on Parafilm that was already attached to a sample target using double-sided carbon tape. After the sol-gel film had formed on the Parafilm (which took ca. 30 min), a mixture (0.2 μ L) of matrix and oligonucleotide (dissolved in water) was applied to the top of the thin film. Matrices were pre-

pared in DMF (30 or 15 mg/mL). The sample was placed into the mass spectrometer once the solvent had evaporated. Additionally, the mixture of the oligonucleotide and matrix was deposited directly onto Parafilm for MALDI analysis to allow a comparison to be made with the results obtained using the sol-gel substrate approach.

Instrument

The experiments were performed using a Biflex III (Bruker Daltonics, Germany) linear time-of-flight mass spectrometer. The mass spectrometer was equipped with a 337-nm nitrogen laser, a 1.25-m flight tube, and a sample target with the capacity to load 384 samples simultaneously. The accelerating voltage was set to 19 kV.

RESULTS AND DISCUSSION

Figure 1(a) displays the MALDI mass spectrum of an oligonucleotide, d(ATG)₄ (6.7 pmol), obtained after depositing equal volumes (0.1 μ L) of the analyte and SA (15 mg/mL) solutions directly onto a sol-gel/2-hydroxymethyl[15]crown-5 hybrid film; the concentration of sodium ions in the oligonucleotide sample was ca. 250 ppm, as determined by inductively coupled plasma mass spectrometry (ICPMS). The $[\text{M}-\text{H}]^-$ ion of d(ATG)₄ dominates the mass spectrum.

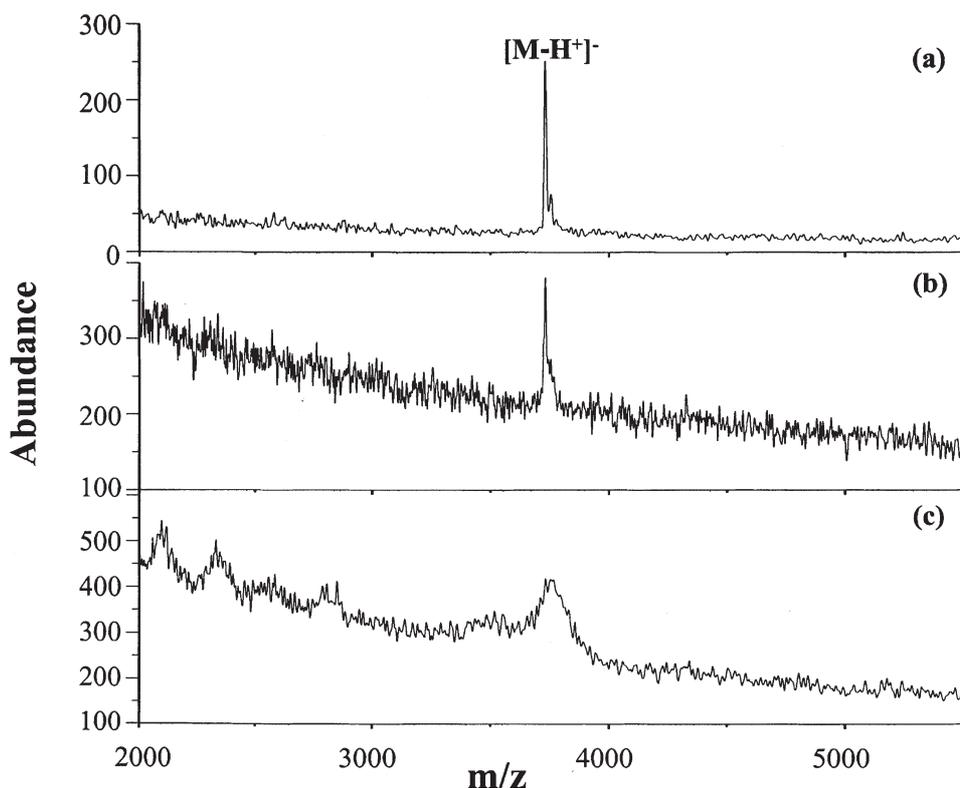


Figure 1. MALDI mass spectra of d(ATG)₄ (6.7 pmol) obtained after depositing a mixture of the analyte with SA (15 mg/mL) directly on (a) a sol-gel/2-hydroxymethyl[15]crown-5 (0.3 M) hybrid film (the mass spectrum was obtained as the sum of 140 laser shots, and mass spectral resolution is 315); (b) a sol-gel/2-hydroxymethyl[18]crown-6 (0.3 M) hybrid film (the mass spectrum was obtained summing 214 laser shots, and mass spectral resolution is ca. 264); and (c) Parafilm (the mass spectrum was obtained summing 167 laser shots, and mass spectral resolution is ca. 18).

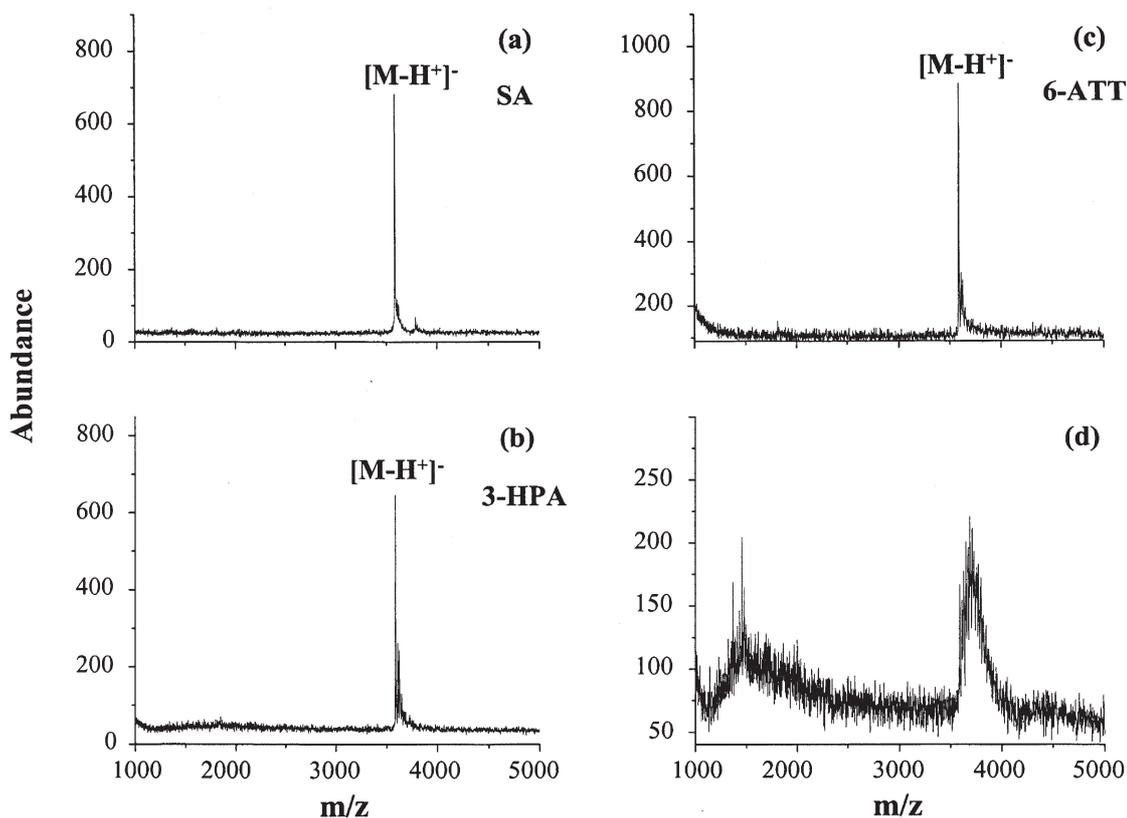


Figure 2. MALDI mass spectra of $d(T)_{12}$ (557 fmol) obtained after depositing a mixture of the analyte with (a) SA (15 mg/mL) directly on a sol-gel/2-hydroxymethyl[15]crown-5 (0.1 M) hybrid film (the mass spectrum was obtained summing 8 laser shots and mass spectral resolution is ca. 639); (b) 3-HPA (15 mg/mL) directly on a sol-gel/2-hydroxymethyl[15]crown-5 (0.1 M) hybrid film (the mass spectrum was obtained summing 31 laser shots and mass spectral resolution is ca. 618); (c) 6-ATT (15 mg/mL) directly on a sol-gel/2-hydroxymethyl[15]crown-5 (0.2 M) hybrid film (the mass spectrum was obtained summing 33 laser shots and mass spectral resolution is ca. 668); and (d) SA (15 mg/mL) directly on Parafilm (the mass spectrum was obtained summing 56 laser shots).

Figure 1(b) presents the corresponding MALDI mass spectrum of $d(ATG)_4$ obtained using a sol-gel/2-hydroxymethyl[18]crown-6 hybrid film as the sample substrate. The $[M-H]^-$ ion of $d(ATG)_4$ still dominates this mass spectrum, but its intensity is slightly lower than that observed in Fig. 1(a). This finding is not surprising because [15]crown-5, whose cavity size is a good fit for a sodium ion, binds Na^+ more strongly than does [18]crown-6, which is slightly too large for such ions.¹⁷ Thus, we used the former crown ether for the remaining experiments reported herein. In the absence of the sol-gel/crown ether substrate, we observe a broad peak representing the unresolved sodium adduct ions of $d(ATG)_4$ (Fig. 1(c)). Furthermore, fragments of $d(ATG)_4$ appear in the lower-mass region. These spectra clearly indicate that use of the sol-gel/crown ether mixture as a substrate is an effective method for reducing the extent of sodium ion adduction of oligonucleotides during MALDI analysis. If the crown ethers are mixed directly with the MALDI matrix, however, the signal for the sodium ion adducts of oligonucleotides still appears in the MALDI mass spectrum. It is possible that poor co-crystallization of the MALDI matrix with analytes arises from the presence of the crown ethers which, therefore, degrades the quality of the spectra. This problem can be avoided simply by depositing the mixture

of analytes and MALDI matrix onto the sol-gel/crown ether hybrid material. SA is a frequently used matrix for MALDI analysis of peptides and large proteins, but is not a common MALDI matrix for oligonucleotide analysis. However, the results show that SA is a suitable matrix in this sol-gel approach.

The common MALDI matrices for oligonucleotide analysis, such as 3-HPA and 6-ATT, were also investigated by this approach. Figures 2(a), 2(b) and 2(c) show the MALDI mass spectra of $d(T)_{12}$ (557 fmol), obtained directly from the sol-gel/2-hydroxymethyl[15]crown-5 hybrid substrate using SA, 3-HPA and 6-ATT as the MALDI matrix, respectively. The $[M-H]^-$ ions of $d(T)_{12}$ dominate these mass spectra. Figure 2(d) presents the MALDI mass spectrum of $d(T)_{12}$ (557 fmol) obtained directly from Parafilm using SA as the MALDI matrix. A very broad peak, corresponding to deprotonated and unresolved sodium adducts of $d(T)_{12}$, was observed. These results indicate that this sol-gel substrate is again effective in desalting when these matrices are employed.

Figure 3(a) displays the MALDI mass spectrum of $d(T)_{24}$ (6.9 pmol) obtained after depositing equal volumes (0.1 μ L) of the analyte (0.5 mg/mL) and SA (30 mg/mL) solutions directly onto the sol-gel/2-hydroxymethyl[15]crown-5 hybrid film. The $[M-H]^-$ ions of $d(T)_{24}$ dominate the mass

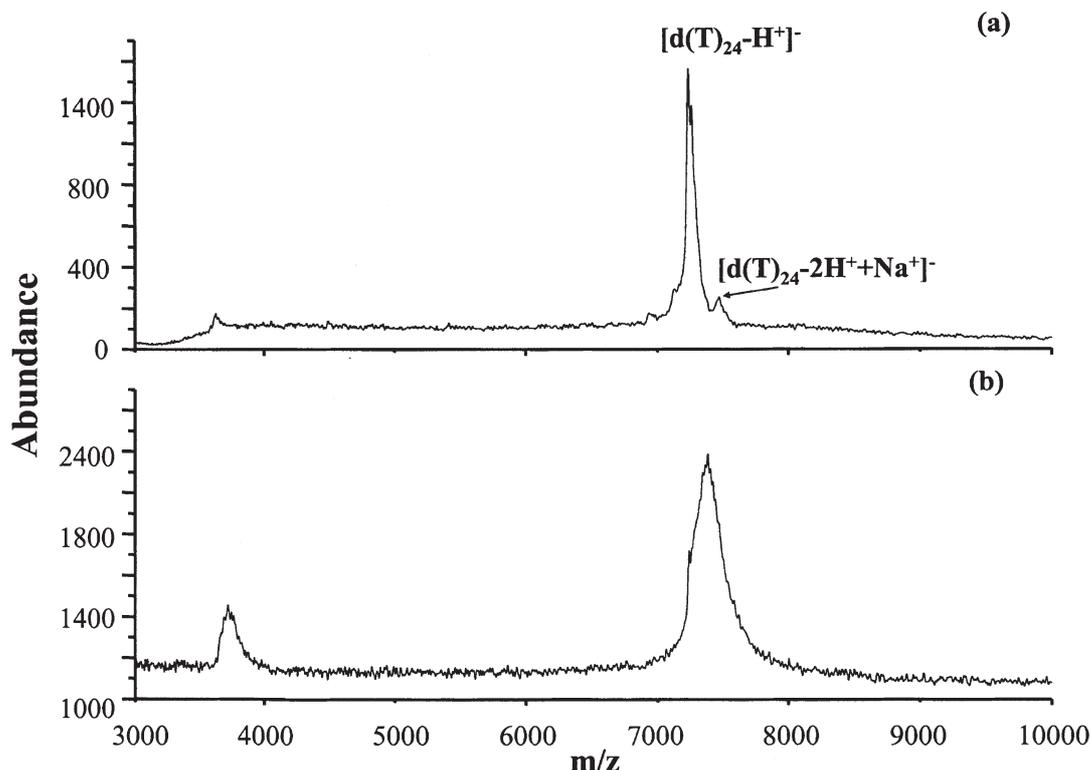


Figure 3. MALDI mass spectra of $d(T)_{24}$ (6.9 pmol) obtained after depositing a mixture of the analyte with SA (30 mg/mL) on (a) a sol-gel/2-hydroxymethyl[15]crown-5 (0.3 M) hybrid film (the mass spectrum was obtained summing 35 laser shots and mass spectral resolution is ca. 112) and (b) Parafilm (the mass spectrum was obtained summing 235 laser shots and mass spectral resolution is ca. 53).

spectrum. Figure 3(b) presents the MALDI mass spectrum of the same sample applied directly to the Parafilm without using the sol-gel/2-hydroxymethyl[15]crown-5 hybrid film as the substrate; the signal of the sodiated oligonucleotide, $[d(T)_{24}-2H^++Na^+]^-$, dominates the mass spectrum. The peak for the $[d(T)_{24}-H]^-$ ion in Fig. 3(a) is obviously much sharper than that of the $[d(T)_{24}-2H^++Na^+]^-$ ion in Fig. 3(b). These results again indicate the effectiveness of the sol-gel/2-hydroxymethyl[15]crown-5 hybrid film as a desalting agent.

Figure 4(a) displays the MALDI mass spectrum of $d(T)_{60}$ obtained after depositing equal volumes (0.1 μ L) of the analyte (0.5 mg/mL) and SA (30 mg/mL) directly onto the sol-gel/2-hydroxymethyl[15]crown-5 hybrid film. Higher laser power was used to obtain this mass spectrum. Thus, in addition to the $[M-H]^-$ ion and the doubly charged ion $[d(T)_{60}-2H]^2-$, a series of y_n fragment ions, each separated by 304 Da (at m/z 5716, 6020, 6324, etc.), appears in the lower-mass region. However, when $d(T)_{60}$ is mixed directly with the MALDI matrix without using the sol-gel/2-hydroxymethyl[15]crown-5 hybrid film as the sample substrate, we observed only a very weak and broad peak for $d(T)_{60}$ in the corresponding MALDI mass spectrum (Fig. 4(b)).

The largest oligonucleotide detectable using this approach in MALDI analysis is close to the 100-mer (see Fig. 5(a)). The $[d(T)_{100}-H]^-$ ion dominates the MALDI mass spectrum and we also observe a peak corresponding to the doubly charged ion. On depositing the analyte directly onto Parafilm without using the sol-gel/2-hydroxymethyl[15]crown-5 hybrid film

as the sample substrate, we observe a broad and weak $[d(T)_{100}-H]^-$ peak (Fig. 5(b)). The centroid of this $[M-H]^-$ peak is shifted to a higher mass relative to that observed in Fig. 5(a).

Figure 6 displays the MALDI mass spectrum of $d(T)_{36}$ (23 fmol) obtained using the sol-gel/crown ether as a substrate; this spectrum suggests that here we approached the detection limit for this approach. High laser power was used to obtain this mass spectrum of $d(T)_{36}$, so the fragment ions were observed in the low-mass region.

To examine whether the sol-gel/crown ether approach combined with matrices other than SA is also suitable for mixed-base oligonucleotide analysis, 3-HPA was used as the matrix for the analysis of a mixed-base oligonucleotide (5'-GATGGAGCCGTAGACCGTCGGTGGAAC-3'). Figure 7 presents the MALDI mass spectrum of this oligonucleotide obtained using the sol-gel/2-hydroxymethyl[15]crown-5 hybrid film as the sample substrate and 3-HPA used as the MALDI matrix. The $[M-H]^-$ ion of the oligonucleotide at m/z 9335 dominates the mass spectrum, and a potassium ion adduct, $[M-2H^++K^+]^-$ at m/z 9373, is also observed. A series of fragment ions, separated by 289 Da (at m/z 4317, 4606, and 4895), was identified as z_n ions based on mass calculations. No analyte-related ions could be observed in the MALDI mass spectrum without using the sol-gel/2-hydroxymethyl[15]crown-5 hybrid film as the sample substrate. These results indicate that this sol-gel/crown ether approach is also suitable for the MALDI-MS analysis of larger mixed-base oligonucleotides.

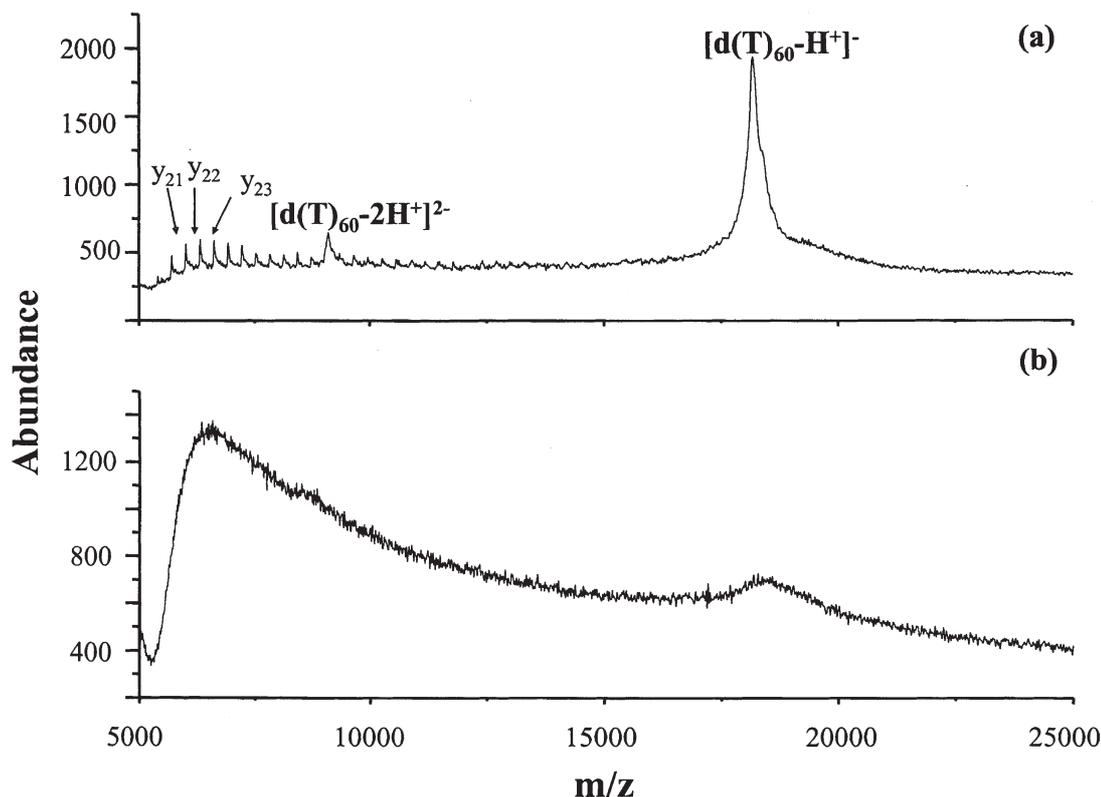


Figure 4. MALDI mass spectra of $d(T)_{60}$ (2.7 pmol) obtained after depositing a mixture of the analyte with SA (30 mg/mL) on (a) a sol-gel/2-hydroxymethyl[15]crown-5 (0.3 M) hybrid film (the mass spectrum was obtained summing 130 laser shots and mass spectral resolution is ca. 120) and (b) Parafilm (the mass spectrum was obtained summing 269 laser shots, and mass spectral resolution is ca. 7).

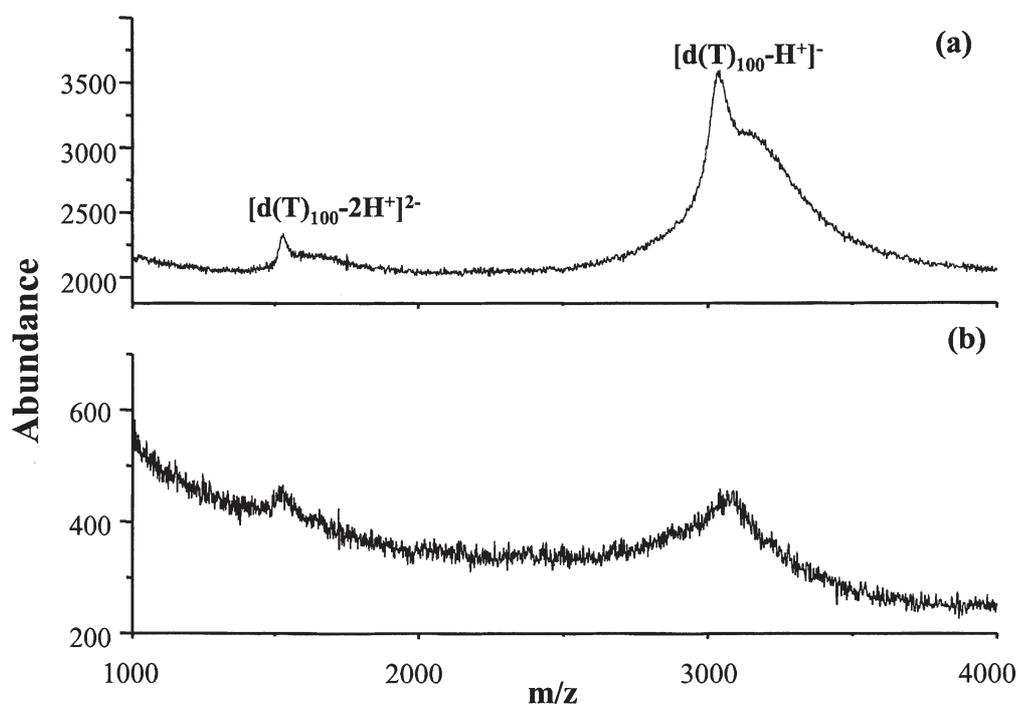


Figure 5. MALDI mass spectra of $d(T)_{100}$ (820 fmol) obtained after depositing a mixture of the analyte with SA (30 mg/mL) on (a) a sol-gel/2-hydroxymethyl[15]crown-5 (0.3 M) hybrid film (the mass spectrum was obtained summing 347 laser shots) and (b) Parafilm (the mass spectrum was obtained summing 212 laser shots).

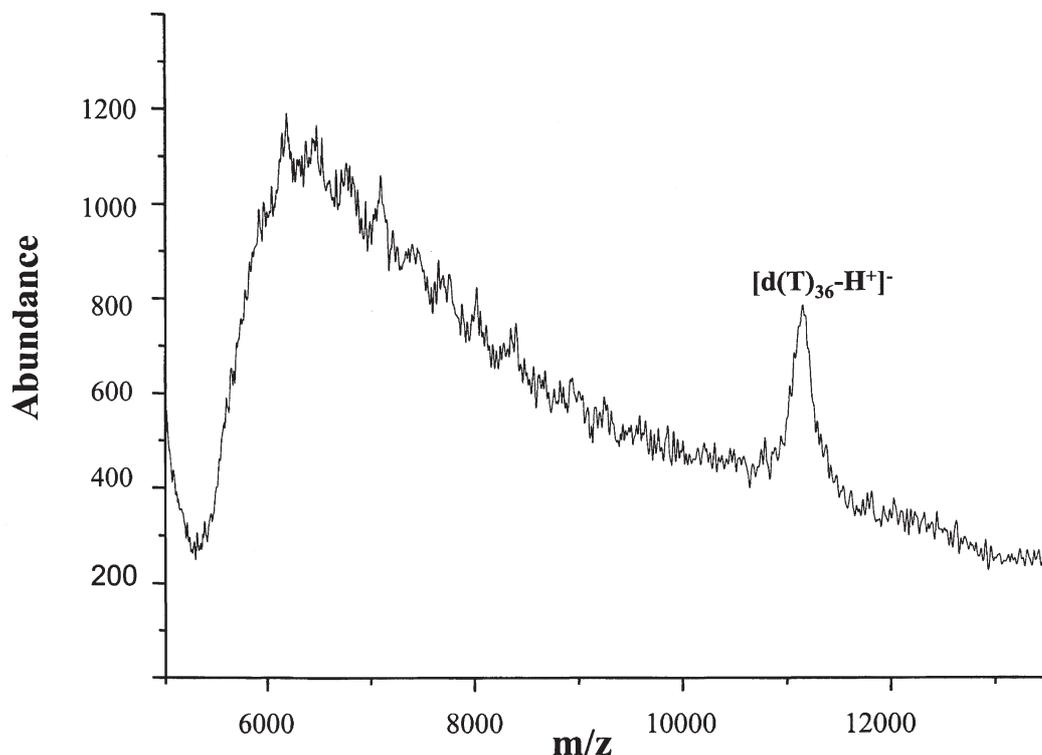


Figure 6. MALDI mass spectrum of $d(T)_{36}$ (23 fmol) obtained after depositing a mixture of the analyte with SA (1.5 mg/mL) on a sol-gel/2-hydroxymethyl[15]crown-5 (0.3 M) hybrid film (the mass spectrum was obtained summing 500 laser shots and mass spectral resolution is ca. 67).

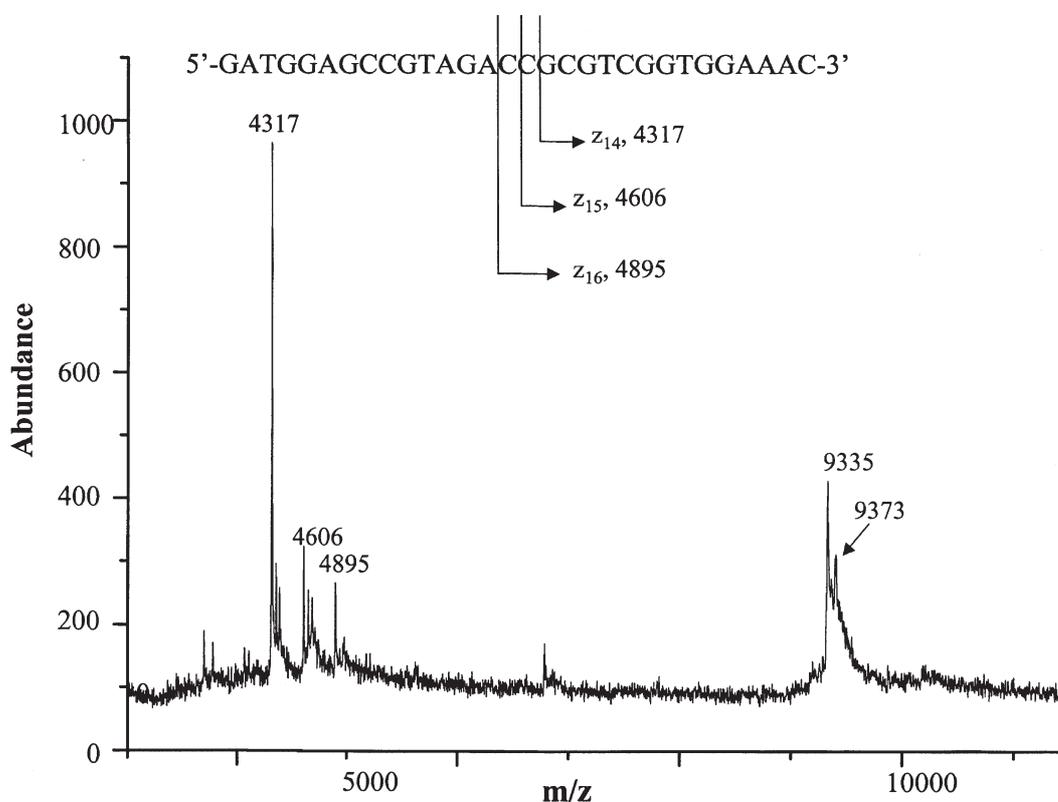


Figure 7. MALDI mass spectrum of 5'-GATGGAGCCGTAGACCGCGTCGGTGGAAAC-3' (5 pmol, $[M-H]^+$ is at m/z 9335) obtained using the sol-gel/2-hydroxymethyl[15]crown-5 (0.1 M) hybrid material as the sample substrate. 3-HPA (saturated solution dissolved in acetonitrile/water (2:1, v/v)) was used as the MALDI matrix (the mass spectrum was obtained summing 160 laser shots, and the mass spectral resolution is ca. 537).

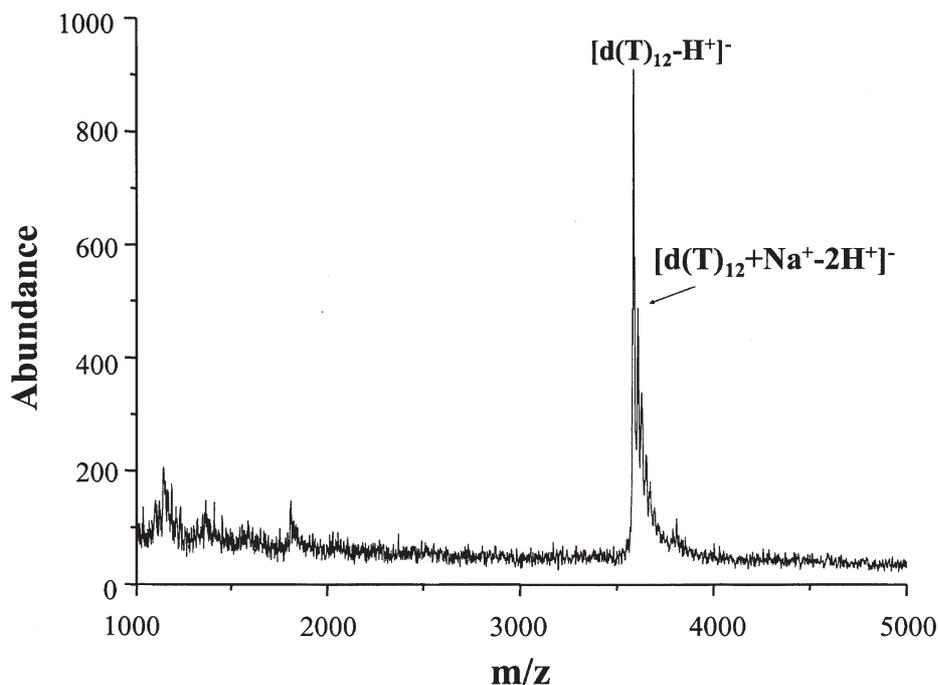


Figure 8. MALDI mass spectrum of $d(T)_{12}$ (1.4 pmol) obtained after the addition of 1% NaCl to the sample solution, using the sol-gel/2-hydroxymethyl[15]crown-5 (0.3 M) hybrid material as the sample substrate. SA was used as the MALDI matrix (the mass spectrum was obtained summing 41 laser shots, and mass spectral resolution is ca. 368).

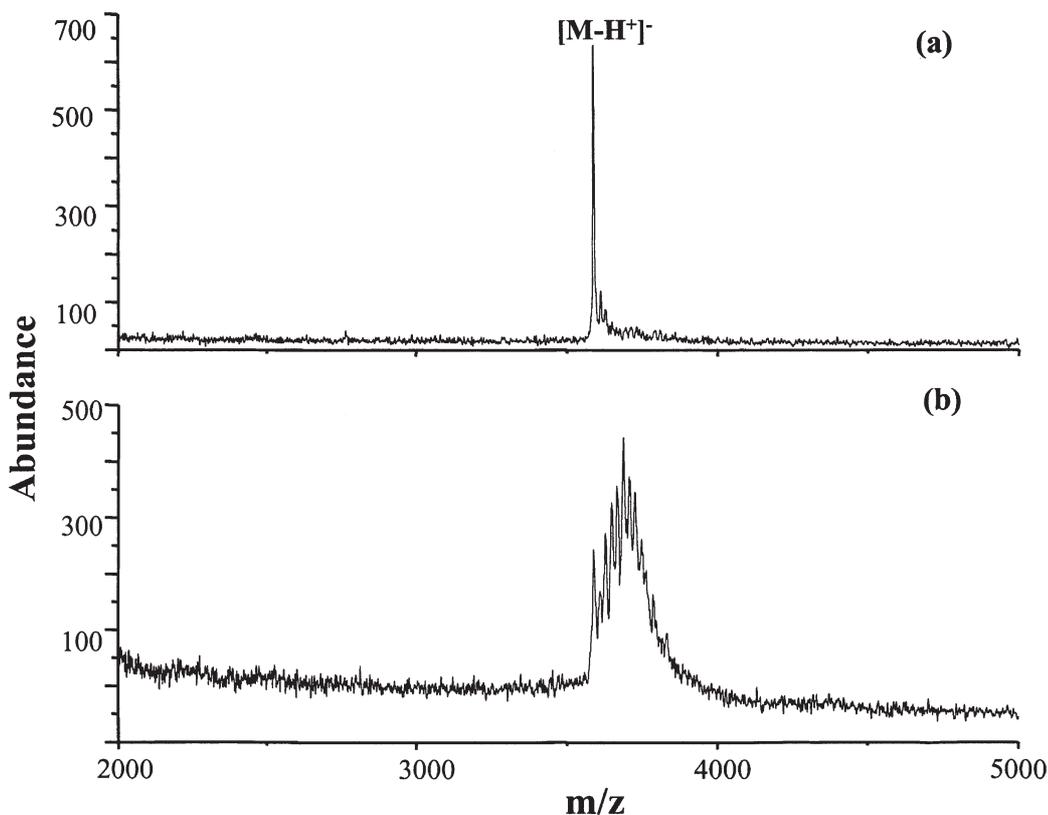


Figure 9. (a) MALDI mass spectrum of $d(T)_{12}$ (1.4 pmol) obtained after incubation of the sample solution with a dried sol-gel/2-hydroxymethyl[15]crown-ether (0.3 M, 20 μ L) hybrid material for 30 min (the mass spectrum was obtained summing 41 laser shots, and mass spectral resolution is ca. 661). (b) Direct MALDI analysis of $d(T)_{12}$ (1.4 pmol) (the mass spectrum was obtained summing 33 laser shots). SA was used as the MALDI matrix in both (a) and (b).

To evaluate the salt tolerance, we spiked a d(T)₁₂ solution with 1% NaCl. Figure 8 presents the MALDI mass spectrum of this spiked d(T)₁₂ sample obtained using the sol-gel/2-hydroxymethyl[15]crown-5 hybrid material. The [d(T)₁₂-H]⁻ ion is the major peak in the mass spectrum. This result indicates that the use of sol-gel/2-hydroxymethyl[15]crown-5 as a substrate for MALDI analysis permits tolerance to salt at concentrations as high as 1% in the sample solution.

The process by which the desalting occurs in this sol-gel approach is of interest. We assume that the sodium ions become trapped by the crown ethers in the sol-gel material. Sodium ions are readily incorporated into the cavities of the crown ethers. To test this assumption we deposited a larger volume (2 μL) of the d(T)₁₂ solution (20 μg/mL) directly onto dried sol-gel/crown ether (20 μL) hybrid material, forming a two-phase system in a capped vial. After standing for 30 min, 0.4 μL of the upper sample solution was mixed with an equal volume of the fresh SA matrix solution (30 mg/mL) for MALDI analysis. Figure 9(a) displays the MALDI mass spectrum of d(T)₁₂ obtained after this treatment. The degree of sodium ion adduction of d(T)₁₂ is effectively reduced relative to that observed (Fig. 9(b)) in the spectrum obtained by direct MALDI analysis of d(T)₁₂. These results suggest very strongly that the sodium ions were eliminated from the sample solution, and presumably transferred into the sol-gel/2-hydroxymethyl[15]crown-5 hybrid material, before MALDI analysis. That is to say, when the upper-phase sample solution used in Fig. 9(a) was mixed with MALDI matrix, the sodium ions had already been removed and the sodium ion adducts of the analyte were thus greatly reduced in the subsequent MALDI mass spectra. These results are consistent with our assumed mechanism for desalting.

CONCLUSIONS

This paper presents a novel means for reducing the extent of sodium ion adductions of oligonucleotides, by simply depositing MALDI samples on a thin layer of sol-gel/2-hydroxy-

methy[15]crown-5 substrate. The substrate functions as a desalting material. The quality of mass spectra of oligonucleotides can be dramatically improved in this way without altering the method of sample preparation used for MALDI analysis.

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