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A RAPID LC/MS METHOD FOR THE HIGH THROUGHPUT QUALITY CONTROL OF LONG OLIGONUCLEOTIDES

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Introduction

Synthetic oligonucleotides are utilized for many diagnostic and therapeutic purposes. Therefore, their quality control (QC) is imperative for monitoring the reliability of synthesis. Traditionally, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) has been used for this purpose; however, oligonucleotides greater than 50mer in length are difficult to analyze, due to decreasing signal intensity and mass accuracy (Fig. 1).

A capillary liquid chromatography/mass spectrometry (LC/MS) method was recently developed for the sensitive analysis and characterization of long oligonucleotides. Duty cycle times were in the range of 20-40 minutes per sample, depending on the size of the oligomer (Fig. 5A). The method proved to be sensitive at subpicomole levels, and was shown to have an average mass accuracy of 125 ppm for oligonucleotides up to 110mer in length. The method was successful for the separation and characterization of guanine-rich oligonucleotides and phosphorothioate (PS) drugs, and their failure sequences/metabolites. However, for routine QC of oligonucleotides, faster analysis is desirable.

Here, an alternative rapid LC/MS method is presented for the high throughput quality control of synthetic oligonucleotides up to 110mer. The method consists of two alternating isocratic LC runs (one for desalting and the other for elution) controlled by a dual position switching valve (Figs. 2 and 3). Duty cycle times for the current method are as low as 1.5 minutes per sample (Fig. 4). This allows for the analysis of approximately 950 samples per 24-hour time period, which is suitable for medium to high throughput applications. The developed method utilizes LC only as a desalting tool, with separation and synthesis component identification being achieved by mass spectrometry (Fig. 5B). As little as 100 picomoles of sample was needed for analysis, and average mass accuracy was determined to be ~ 80 ppm (Table 1).

Experimental

HPLC System: A Waters® Alliance® 2795 Separations Module served as both the autosampler and elution pump. Elution flow rate was 300 mL/min. Experiments were performed at ambient temperature.

Column: XTerra® MS C_{18}, 10 x 2.1 mm, 3.5 mm (guard column)

Isocratic pump: A Waters 515 HPLC pump was plumbed through the Alliance® 2795 Separations Module to serve as the load/wash pump. Load/wash flow rate was 900 mL/min. Salts eluting from the guard column were diverted to waste via a switching valve during the load/wash phase of the LC/MS method.

MS: An orthogonal ESI-ToF mass spectrometer (Waters Micromass® LCT™) was connected in-line to the HPLC system and isocratic pump via a Rheodyne® 6-port, 2-position, stainless steel switching valve.

MS conditions: Capillary 2500 V; sample cone 25 V; desolvation temperature 275 °C; source temperature 120 °C; MCP 2700 V; desolvation gas flow rate 410 L/hr.; cone gas flow rate 30 L/hr. Acquisition range was m/z 500-2000. The 0.95 s scan cycle consisted of a 0.9 s acquisition time and a 0.05 s delay.

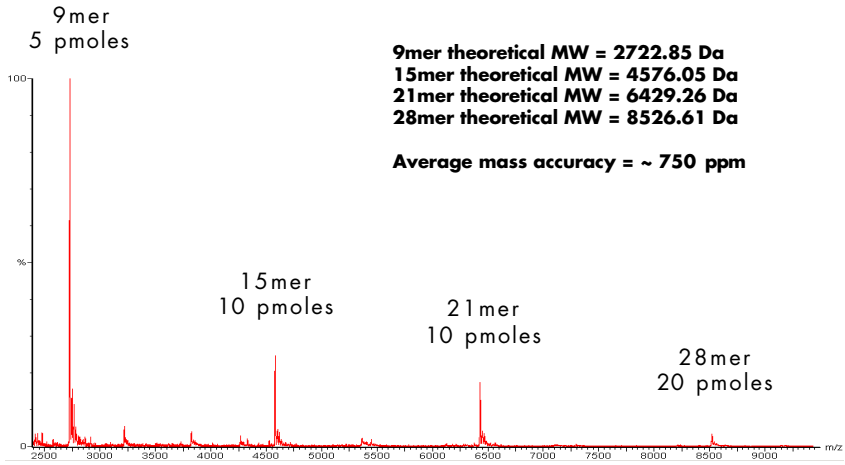
Mobile phases: Load/wash buffer consisted of 5% acetonitrile and 95% 5 mM dimethylbutylammonium acetate (DMBAA), pH 7. Elution mobile phase consisted of 25% acetonitrile and 75% 5 mM DMBAA, pH 7.

Samples: Synthetic oligonucleotides ranging in length from 24mer to 110mer were purchased from the vendor and analyzed without purification.

Software: The system was operated by Waters MassLynx™ Software, Version 3.5. Raw spectra were deconvoluted using the MaxEnt1™ option.

Duty cycle: The rapid LC/MS method consisted of 15 seconds loading and washing, 53 seconds elution, and 22 seconds re-equilibration.

Results and Discussion



Conditions: Oligonucleotides desalted by SPE prior to analysis and mixed 1:1 with 25 mg/mL 3-HPA matrix in 25% ACN: 75% dH_2O. MALDI-ToF analysis performed in positive linear mode. Pulse 1200 V; source 15000 V; MCP 1850 V; matrix suppression delay 500 amu.

Figure 1: The sensitivity and resolution of MALDI-ToF MS rapidly decrease with increasing oligonucleotide length. The calculated limit of detection (LOD) of MALDI for the 9mer oligonucleotide is ~ 30 fmol, while the LOD for 28mer is ~ 580 fmol.

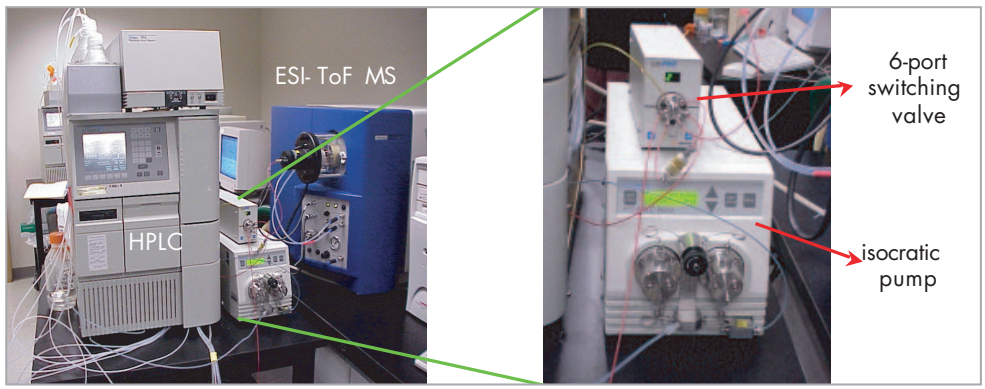


Figure 2: Rapid LC/MS System Configuration.

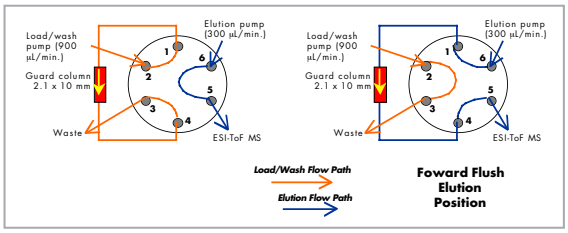


Figure 3: Configuration of switching valve for on-line desalting of oligonucleotides.

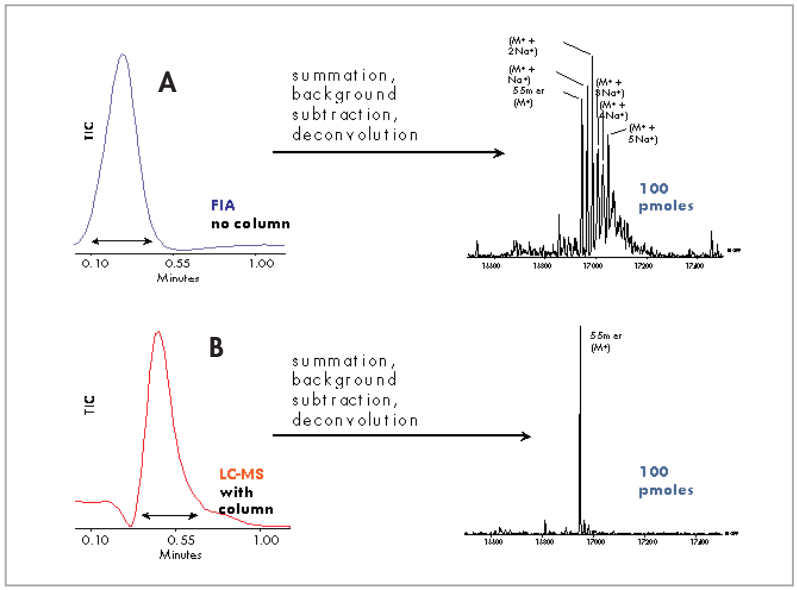


Figure 4: Comparison of (A) flow injection analysis (FIA) and (B) rapid LC/MS for electrospray ionization-mass spectrometry (ESI-MS) analysis of a 55mer oligonucleotide (theoretical MW = 16946.10 Da). For FIA experiments, oligonucleotides were reconstituted in 50% acetonitrile: 50% dH2O only. More than 5 sodiated oligonucleotide forms can be observed by flow injection analysis for 55mer (Fig. 4A). Conversely, sodium adducts were dramatically reduced by rapid on-line LC desalting prior to MS (Fig. 4B).

Oligonucleotide Length	Injected Mass (nmoles)	Theoretical MW (Da)	Experimental Mass Difference (Da)	Mass Accuracy (ppm)
25mer	0.1	7680.07	-0.74	96
35mer	0.1	10744.06	0.63	59
40mer	0.1	12293.07	1.08	88
55mer	0.1	16946.10	1.53	89
70mer	0.1	21559.10	0.42	20
80mer	0.5	24672.13	2.80	114
90mer	1.0	27736.12	2.23	80
100mer	1.0	30825.13	2.02	65
110mer	3.0	33938.16	1.69	50

Table 1: Rapid LC/MS quality control of 25-110mer synthetic oligonucleotides. Over 20 different synthetic oligonucleotides of unique sequence and length were analyzed. The average mass accuracy obtained for all LC/MS experiments was approximately 80 ppm. As little as 100 picomoles was needed for LC/MS analysis, which afforded excellent signal-to-noise ratios.

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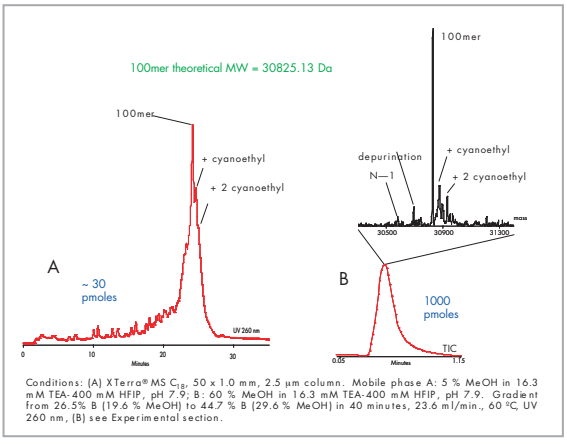

Conditions: (A) XTerra® MS C_{18}, 50 x 1.0 mm, 2.5 µm column. Mobile phase A: 5 % MeOH in 16.3 mM TEA-400 mM HFIP, pH 7.9; B: 60 % MeOH in 16.3 mM TEA-400 mM HFIP, pH 7.9. Gradient from 26.5% B (19.6 % MeOH) to 44.7 % B (29.6 % MeOH) in 40 minutes, 23.6 ml/min., 60 °C, UV 260 nm, (B) see Experimental section.

Figure 5: Comparison of (A) capillary LC/MS, and (B) rapid LC/MS for the quality control of the same crude 100mer synthetic heterooligonucleotide. Separation and characterization of long oligonucleotides and their synthesis impurities (cyanoethyl is a protection group used in synthesis) was achieved by high resolution capillary LC/MS (Fig. 5A). The current high throughput LC/MS method relies on the resolving power of orthogonal ESI-ToF mass spectrometry for separation of the target product from synthesis impurities (Fig. 5B).

Conclusions

- Sample preparation (desalting) is critical for the success of both ESI-MS and MALDI analysis of oligonucleotides.
- On-line desalting by LC prior to MS is highly efficient.
- A rapid LC/MS method has been designed for medium to high throughput QC of oligonucleotides. Approximately 950 samples per 24-hour time period can be analyzed.
- Rapid LC/MS (1.5 minute duty cycle time) is well suited for analysis of long oligonucleotides (> 50mer) that cannot be easily analyzed by MALDI-MS.
- The method showed ~10-fold improvement in average mass accuracy when compared to linear MALDI-ToF MS (80 ppm versus 750 ppm).
- The ESI-ToF mass spectrometer can resolve the target oligonucleotide from its synthetic impurities.
- ESI-MS can be used for purity confirmation and assessment of synthetic oligonucleotides.
- The developed on-line desalting method is applicable for rapid clean up of PCR samples and SNP genotyping fragments.

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