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Rapid LC-MS Analysis of Oligonucleotides: Application to Genotyping and Quality Control

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Overview

- An LC-MS method with a duty cycle of 1.5 minutes per sample has been developed for the rapid QC of synthetic oligonucleotides.
- The method is well-suited for analysis of long oligonucleotides (> 50mer) that cannot be easily analyzed by MALDI-TOF MS.
- The method can also be utilized for the analysis of PCR products and SNP genotyping fragments.

Introduction

Synthetic oligonucleotides are utilized for many diagnostic and therapeutic purposes. Therefore, 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 [1]. Routine separation and identification of guanine-rich oligonucleotides and phosphorothioate (PS) drugs was also achieved. Duty cycle times were in the range of 20-40 minutes per sample, depending on the size of the oligomer (Fig. 5A). However, for routine QC of oligonucleotides, faster analysis is desirable.

Here, an alternative rapid LC-MS method is presented for the high throughput QC 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. 4B). 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 MS (Fig. 5B). As little as 100 picomoles of sample were needed for analysis, and average mass accuracy was determined to be 80 ppm (Table 1). The method was also utilized for analysis of PCR products and single nucleotide polymorphism (SNP) genotyping fragments (Fig. 6).

Experimental

HPLC System: An Alliance® 2795 system (Waters Corporation, Milford, MA, USA) served as both the autosampler and elution pump. Elution flow rate was 300 µl/min. Experiments were performed at ambient temperature.

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

Isocratic pump: A 515 HPLC pump (Waters) was plumbed through the Alliance® 2795 HPLC system to serve as the load/wash pump. Load/wash flow rate was 900 µl/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 (Micromass® LCT™, Waters) was connected in-line to the HPLC system and isocratic pump via a Rheodyne® 6-port, 2-position, stainless steel switching valve (Waters). The system was operated by Micromass® MassLynx™ software, version 3.5 (Waters). Raw spectra were deconvoluted using the MaxEnt1™ option.

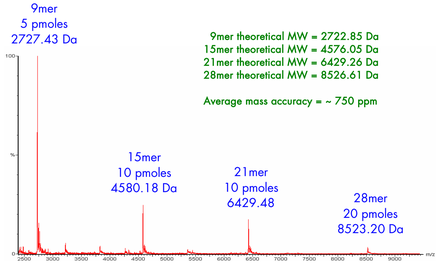
MS Conditions: Capillary 2500 V; sample cone 25 V; desolvation temperature 275 °C; source temperature 120 °C; MCP 2700 V; desolvation gas flow rate 410 L/hr.; cone gas flow rate 30 L/hr. Acquisition range was *m/z* 500-2000. The 0.95 s scan cycle consisted of a 0.9 s acquisition time and a 0.05 s delay.

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.

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

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.



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 MS analysis performed in positive linear mode. Pulse 1200 V; source 15000 V; MCP 1850 V; matrix suppression delay = 500 amu.

Figure 2: Rapid LC-MS System Configuration

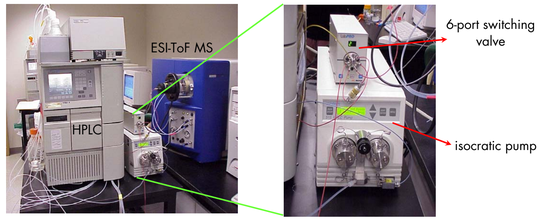


Figure 3: Configuration of switching valve for on-line desalting of oligonucleotides

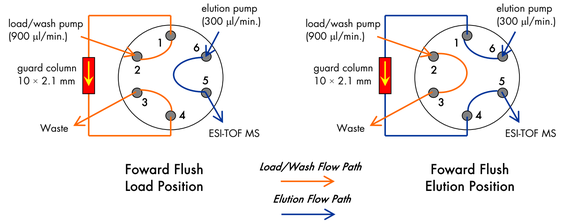


Figure 4: Comparison of (A) flow injection analysis (FIA) and (B) rapid LC-MS for analysis of a 55mer oligonucleotide. More than 5 sodiated oligonucleotide forms can be observed by FIA (Fig. 4A). Sodium adducts were dramatically reduced by rapid on-line LC desalting prior to ESI-MS (Fig. 4B).

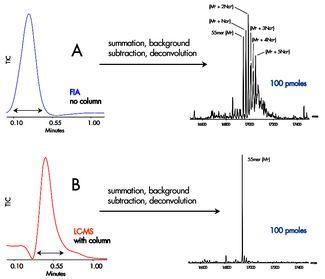
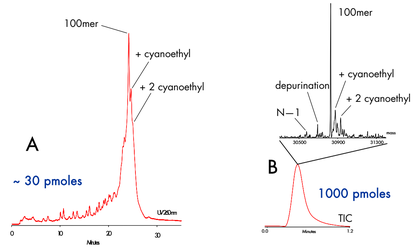


Table 1: Rapid LC-MS QC of 25-110mer synthetic oligonucleotides. Average mass accuracy obtained for all LC-MS experiments was 80 ppm. As little as 100 picomoles was needed for analysis, which afforded excellent signal-to-noise ratios.

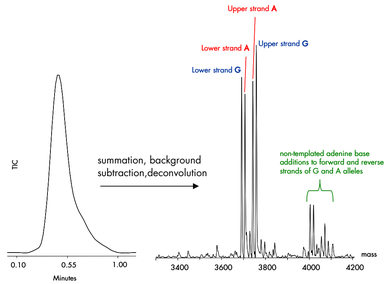
Oligonucleotide Length	Injected Mass (nmoles)	Theoretical MW (Da)	Experimental Mass Difference (Da)	Mass Accuracy (ppm)
25mer	0.1	7680.07	-0.74	96
35mer	0.1	10744.06	0.63	59
40mer	0.1	12293.07	1.08	88
55mer	0.1	16946.10	1.53	89
70mer	0.1	21559.10	0.42	20
80mer	0.5	24672.13	2.80	114
90mer	1.0	27736.12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

Figure 5: Comparison of (A) capillary LC-MS, and (B) rapid LC-MS for the QC 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 MS for separation of the target product from synthesis impurities (Fig. 5B).



Conditions: (A) XTerra® MS C_{18}, 50 × 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 µl/min., 60 °C, UV 260 nm, (B) see Experimental section.

Figure 6: Rapid LC-MS analysis of a SNP genotyping sample that is heterozygous (G/A) at nucleotide 196 for the brain-derived neurotrophic factor (BDNF) protein. The polymorphism of guanine (G) to adenine (A) at this site results in a valine (V) to methionine (M) amino acid change at position 66 in the protein. This SNP has been associated with human hippocampal function [2] and pathogenesis of Parkinson's disease [3].



Conditions: SNP genotyping fragments were generated by PCR and isothermal amplification according to the published protocol [4]. The BDNF protein SNP generated 12mer oligonucleotides during the isothermal amplification step. Forward primer sequence was (5'-3') TGA CAT CAT TGG CTG AGT CTT TCG A. Reverse primer sequence was (5'-3') TTC TGG TCC TCA TCC AAG AGT CCT TCT A. "Upper strand" and "lower strand" refer to the forward and reverse strands, respectively, generated by isothermal amplification.

Conclusions

- Sample preparation (desalting) is critical for the success of both ESI- and MALDI MS analysis of oligonucleotides.
- On-line desalting by LC prior to ESI-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 routine purity confirmation and assessment of synthetic oligonucleotides.
- The on-line desalting method is applicable for rapid clean up of PCR samples and SNP genotyping fragments.

References

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[2] M.F. Egan, M. Kojima, J.H. Callicott, T.E. Goldberg, B.S. Kolachana, A. Bertolino, E. Zaitsev, B. Gold, D. Goldman, M. Dean, B. Lu, D.R. Weinberger, *Cell* 112 (2003) 257.
[3] Y. Momose, M. Murata, K. Kobayashi, M. Tachikawa, Y. Nakabayashi, I. Kanazawa, T. Toda, *Ann Neurol* 51 (2002) 133.
[4] J. Van Ness, L.K. Van Ness, D.J. Galas, *Proc Natl Acad Sci USA* 100 (2003) 4504.