

Microchip CE MS of Oligonucleotides: What can it separate?

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Highlights

- Microfluidic CE/MS analysis of Oligos by PATsmart™ ZipChip®/QExactive HF
- The ZipChip Oligos Kit (Bare Glass Chip Oligos BGE) was used for the analysis
- Orthogonal to LC simple sample prep fast separation 5 min)
- No ion-pairing agents required
- Linear range 5 nM to 1 μ M with LOD at 5 nM (unmodified 20mer 1 nL injection Vol)
- Vendor agnostic Compatible with MS from ThermoFisher Bruker, Sciex

Introduction

Oligonucleotides (oligos) represent a distinct class of therapeutics that include RNA, DNA, and their structural analogs that are effective against a wide range of disease conditions. They are single- or double-stranded linear molecules containing nucleotide residues bound together by phosphodiester. These polyanionic molecules exhibit different chemical and physiological properties compared to small molecule and protein-based drugs. Current mass spectrometry technologies for characterization of oligos include MALDI TOF and LC MS (IP-RP and HILIC). These methods either don't provide high resolution accurate mass (HRMS) characterization or involve the use of ion pairing agents, leading long analysis times for column equilibration.

In this study, we present a microfluidic capillary zone electrophoresis-based separation coupled with HRAM mass spectrometry for rapid characterization of oligos in positive ionization mode. The method utilizes an MS-friendly background electrolyte (that eliminates the use of ion pairing agents such as hexafluoroisopropanol or alkyl amines). Short analysis times coupled with no instrument downtime for decontamination makes this method attractive for high-throughput analysis of oligonucleotides.

Materials & Methods

Samples: Synthetic ssRNA standards of varying sizes (10, 20, and 40) and modifications (unmodified phosphorothioate, phosphorylation, biotinylation, and glycan spacer) were purchased from Integrated DNA Technologies, with dried samples present in each vial. All standards were reconstituted with the Oligos BGE to obtain 500 nM as stock solutions.

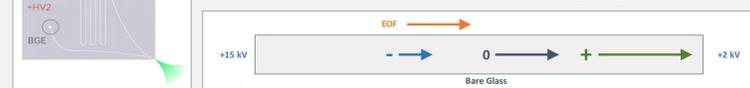
Sample Preparation and Tests: Separation: A 500 nM mixture of equal concentrations of a fully phosphorothioated 20mer RNA along with other modifications such as phosphorylation, biotinylation and glycan spacer was used. **Dynamic range:** Unmodified 10, 20, and 40mer were serially diluted up to 1 nM, 5 nM, and 50 nM respectively with the Oligos BGE. **Robustness:** Unmodified 20mer RNA at 1 μ M on three separate HRB chips with 125 injections per chip.

Instrumentation: The ZipChip Device was used for all analyses.¹ The High Resolution Bare (HRB) Glass Chip and the Oligonucleotides BGE, both part of the Oligonucleotides kit (Repligen) were used. An on-chip injection volume was 1 nL with separation run at a field strength of 500 V/cm. MS analysis was performed on a Thermo Fisher QExactive HF mass spectrometer.



Bare Glass Chip – Separation Mechanism

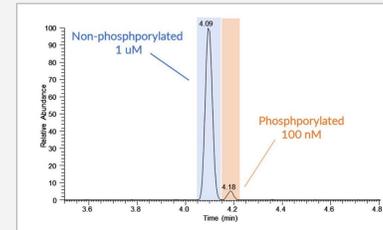
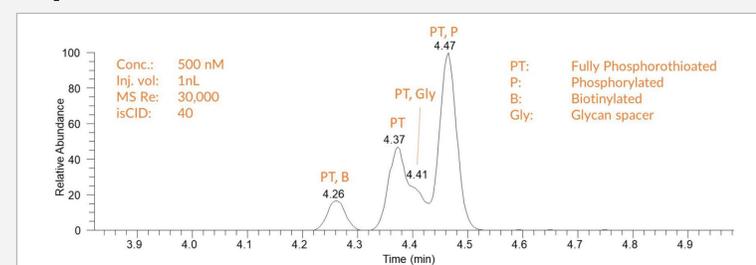
Unlike the standard neutral polymer coated chips, the channels in the HRB chips contain significantly charged bare silica surface. When a voltage potential difference is applied across the separation channel, it induces a steady unidirectional motion of the BGE resulting in electroosmotic flow (EOF) which results in the migration of all analytes towards the ESI sprayer. Positively charged species migrate first, followed by neutrals which in turn are followed by negatively charged species, thus allowing for electrophoretic separation of analytes with varying charges.



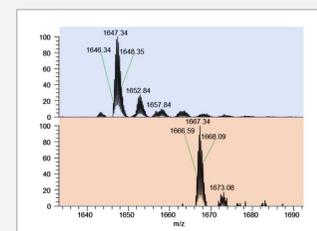
Characterization of Oligonucleotides

The ZipChip CE/HRMS provided a rapid robust analysis for oligos in less than 5 minutes. The method employed the ZipChip Oligos Kit which includes a bare glass chip and Oligos BGE (pH ~8.5). Either the High-Speed Bare Glass Chip (HSB) or the High-Resolution Bare Glass Chip (HRB) can be used based on user preference. For this work, the HRB chip was used. The bare glass chip enabled easy analysis of negatively charged oligonucleotides in short analysis times. Sharp peaks with gaussian distribution were observed and no ion-pairing agents were necessary. Analytes were electrosprayed at a sprayer potential of +3.5 kV and were therefore detected as positively charged ions.

Separation of Modified ssRNA Standards



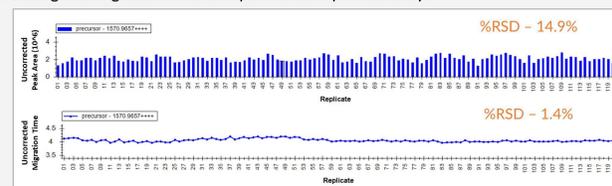
To assess the separation efficiency of the method, several modified 20mer ssRNAs with phosphorothioation at every site were obtained with same sequence - rC*rG*rG*rC*rU*rA*rC*rC*rU*rU*rG*rU*rU*rA*rC*rG*rA*rC*rU*rU. Modifications included phosphorylation, biotinylation and a glycan spacer consisting of 1',2'-dideoxyribose. Phosphorylation and biotin modified RNAs were baseline resolved from the main peak whereas the dideoxyribose modified RNA peak appeared as a shoulder to the main peak as shown in the figure above.



In a separate experiment, 100 nM phosphorylated 20mer ssRNA was spiked into a 1 μ M solution of the corresponding non-phosphorylated 20mer ssRNA standard. Due to the presence of an additional negative charge on the phosphorylated molecule, it detected as a baseline resolved peak from the non-phosphorylated molecule as shown in the above left figure. The resulting mass spectra for the two peaks are also shown to the left. The order of migration was in accordance with the net charge of the molecules with the molecule with less net negative charge migrating first.

Robustness and Reliability

To demonstrate the robustness and reliability of the method, a 1 μ M 20mer ssRNA (unmodified) was analyzed with a total of 375 injections over 3 consecutive days. Three HRB chips were used for this test (125 inj/chip, 1 chip/day), one BGE refresh after every 3 injections. A representative plot from a 125 inj sequence is shown below demonstrating the migration time and peak area reproducibility.



Unmodified 20 and 40mer ssRNA

The figure below displays electropherograms for 1 μ M of unmodified 20mer (top left) and 2 μ M solution of 40mer (bottom left) ssRNAs and their MS spectra (right). An insert in the mass spectra shows a zoomed in view of the [M+4H]⁴⁺ and [M+5H]⁵⁺ species that were detected as the dominant charge states for 20 and 40mer ssRNAs respectively. Orbitrap resolution settings were 30,000 for 20mer and 60,000 for 40mer. Optimized in-source fragmentation energy was 40 for 20mer and 80 for 40mer. In both cases, the protonated adduct was observed to the prominent adduct, along with other minor alkali metal adducts such as sodium and potassium. The level of adducting appeared to be increasing with the size of the oligos.

