Microchip CE-MS of oligonucleotides: What can it separate?

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Highlights

- Microfluidic CE/MS analysis of Oligos by 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 uM with LOD at 5 nM (unmodified 20 mer) (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 phosphodiesters. 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 a MS-friendly background electrolyte (BGE), that eliminates the use of ion-paring agents such as hexafluoro isopropanol or alkyl amines. Short analysis times coupled with no instrument downtime for decontamination makes this method attractive for high-throughput analysis of oligonucleotides.

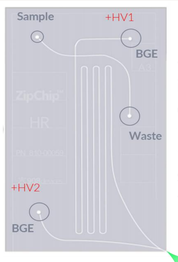
Materials & Methods

Samples: Synthetic ssRNA standards of varying sizes (10, 20 and 40) and modifications (unmodified, phosphorothioate, phosphorylation, biotinylated and glycan spacer) were purchased from Integrated DNA Technologies, with dried samples present in each vial. All standards were reconstituted with the Oligos BGE (908 Devices Inc.) to obtain 500 nM as stock solutions.

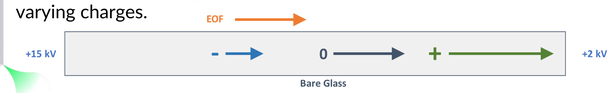
Sample Preparation and Tests: Separation: A 500 nM mixture of equal concentrations of a fully phosphorothioated 20mer RNA along with other modifications such as phosphorylation, biotinylation and glycan spacer was used. **Dynamic range:** Unmodified 10, 20 and 40 mer were serially diluted up to 1 nM,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

Instrumentation: The ZipChip™ Device was used for all analyses (908 Devices Inc.).[1] The High Resolution Bare (HRB) Glass Chip and the Oligonucleotides BGE, both part of the Oligonucleotides kit (908 Devices Inc.) 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,[2] 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.

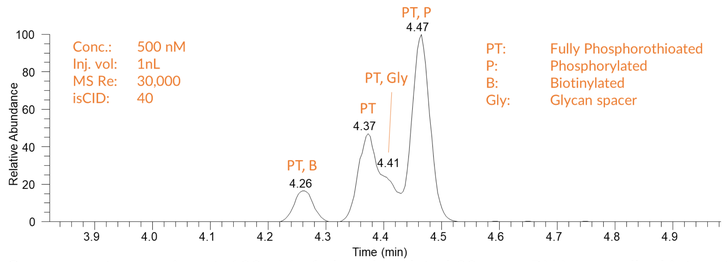


Characterization of Oligonucleotides

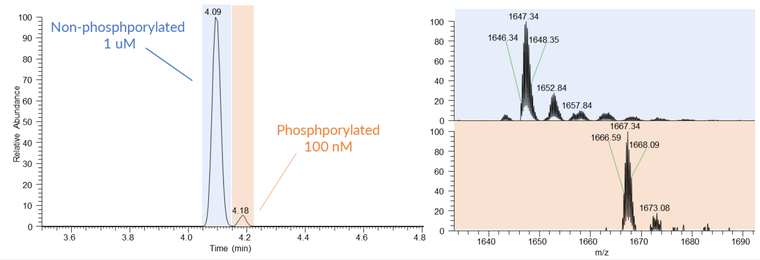
The ZipChip CE/HRMS provided a rapid & robust analysis for oligos in less than 5 minutes. The method employed the ZipChip Oligos Kit which includes a bare glass chip and Oligos BGE (pH ~8.5). Either the High-Speed Bare Glass Chip (HSB) or the High-Resolution Bare Glass Chip (HRB) can be used based on 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 +3.5kV and were therefore detected as positively charged ions.

Separation of Modified ssRNA Standards

To assess the separation efficiency of the method, several modified 20 mer 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 below.

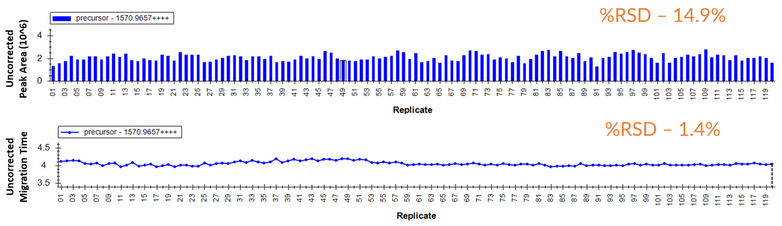


On a separate experiment, 100 nM of phosphorylated 20mer ssRNA was spiked into a 1 uM solution of the corresponding non-phosphorylated 20mer ssRNA standard. Due to the presence of an additional negative charge on the phosphorylated molecule, it detected as a baseline resolved peak from the non-phosphorylated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



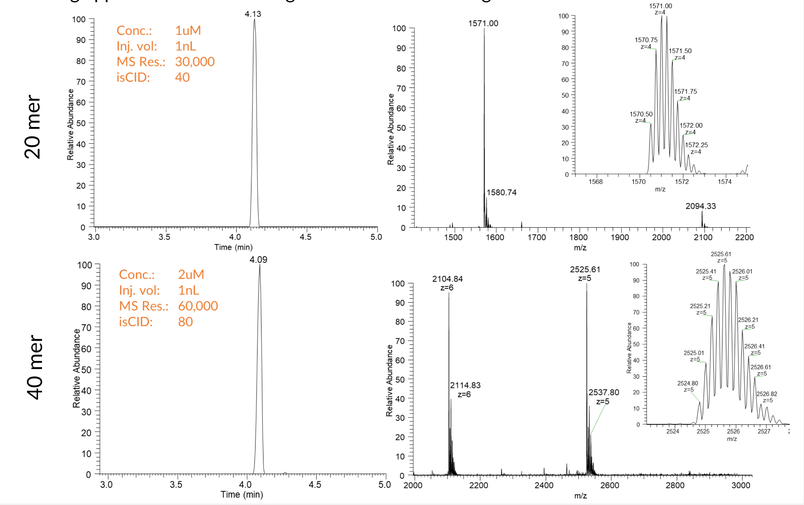
Robustness and Reliability

To demonstrate the robustness and reliability of the method, a 1 uM 20mer ssRNA (unmodified) was analyzed with a total of 375 injections over 3 consultive 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 showing below demonstrating the migration time and peak area reproducibility.



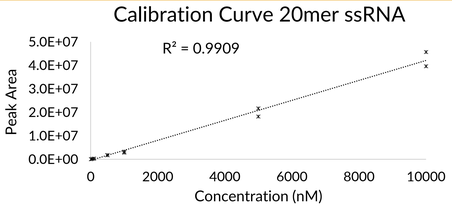
Unmodified 20 and 40mer ssRNA

Figure below displays electropherograms for 1uM of unmodified 20 mer (top left) and 2uM solution of 40 mer (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 40 mer ssRNAs respectively. Orbitrap resolution settings were: 30,000 for 20mer; 60,000 for 40 mer. Optimized in-source fragmentation energy was: 40 for 20 mer, 80 for 40 mer. 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.



Linearity, Dynamic Range and Sensitivity

The sensitivity of the method was tested using the unmodified 20mer ssRNA. The standard was serially diluted in the BGE. Each calibration standard was injected in duplicate. The the calibration curve (right) showed 5 nM -10 uM dynamic range (R^2 0.99). The LOD and LOQ were found to be 5 nM and 10 nM respectively.



Future Work

The ZipChip is MS vendor agnostic, hence future work would involve a full evaluation of the ZipChip oligos kit on Sciex TripleTOF and Bruker's timsTOF mass spectrometers. Efforts will also be focused on more in-depth analysis of impurities, MSMS analysis, forced degradation and Met ID studies of API, and double stranded molecules.

Conclusions

The ZipChip CE/MS enables a simple, sensitive, dilute and shoot method for rapid analysis of oligonucleotides, applicable to various sizes and modifications. The method produces separations that are orthogonal to LC/MS. The method requires NO toxic ion-pairing agents, eliminating instrument down time due to contamination and thus, enables fast switching between different applications. The method exhibits linearity over a wide dynamic range, can separate modified oligonucleotides from the corresponding unmodified oligonucleotides and shows excellent peak area and migration time reproducibility.

References

1. Tech Note 1.0; 908 Devices; *ZipChip: What are they and how they work.*
2. Redman, E. A.; Batz, N. G.; Mellors, J. S.; Ramsey, J. M. *Anal. Chem.* **2015**, *87*, *4*, *2264-2272.*