ZipChip

Rapid characterization of oligonucleotides using microfluidic capillary electrophoresis - mass spectrometry by ZipChip

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KEYWORDS

Oligonucleotides, RNA, DNA, capillary electrophoresis, tandem mass-spectrometry, oligonucleotide modifications, quantitation

BENEFITS

- High resolution accurate mass (HRAM) mass spectrometry allows for accurate identification and quantitation of oligonucleotides.
- CE separations offer an orthogonal analysis method compared to traditional LC-based separation methods.
- Analysis times are short (usually 5-6 min) and the method does not require extensive sample preparation or method optimization.
- No ion-pairing agents are necessary.

INTRODUCTION

Oligonucleotides (oligos) represent a distinct class of therapeutics that include RNA, DNA and their structural analogues, and are effective against a wide range of disease conditions. Synthetic oligos have gained significant interest from the biopharmaceutical industry recently, and several of these candidates have been approved or have progressed to later stages of clinical trials. Oligos 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 and pose a different set of challenges for analytical characterization. Currently, LCMS is still the gold standard for the characterization of oligos, especially widely used is the reverse phase LC using ion pairing (IP) reagents. These methods not only involve the use of ion-pairing agents, but also require long analysis times for system and column equilibration. It is typical that a dedicated LC/MS system is required for the analysis of oligos. An orthogonal separation method coupled with high resolution mass spectrometry that offers simple and easy analysis of the Oligos is highly desirable.

The ZipChip® device is a front inlet of a mass spectrometry. It enables capillary zone electrophoresis (CZE) based separation that harnesses the inherent speed of microfluidic technology to improve the efficiency of CE separations. The ZipChip device can be seamlessly coupled to variety of commercial mass spectrometers from Thermo Fisher Scientific, Bruker and SCIEX.

In this work, we present a CE/MS analysis of oligos by ZipChip coupled to a QExactive HF mass spectrometer. This positive ESI mode method utilizes a MS-friendly background electrolyte (BGE), that eliminates the use of ion-paring agents such as hexafluoro isopropanol or alkyl amines. This ensures fast switching time between methods, no downtime associated with instrument decontamination. The analysis times are short (typically < 5) with minimum method development required.

ZipChip mounted to a Thermo Scientific™ Q Exactive HF-X mass spectrometer.

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MATERIALS AND METHODS

Reagents and ZipChip Consumables:

The ZipChip Oligos Kit (908 Devices Inc.; p/n 850-00066) was used for all analyses. The High Resolution Bare Glass (HRB) (p) (908 Devices Inc.; p/n 810-00239) chips were used for sample analysis by the ZipChip.

Samples:

Custom synthesized 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.; p/n 850-00066) to obtain 500 uM stock solutions. Standards were further diluted in the BGE as needed.

Tests Performed

Separation:

A 500 nM mixture of equal concentrations of a fully phosphorothioated 20 mer 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 20 mer RNA standard was analyzed at a concentration of 1 uM on three separate HRB chips with 125 injections per chip.

Instruments:

A classic ZipChip interface (908 Devices Inc.; p/n ZCIA-01-0-0-01) was used as the microfluidic CZE inlet. The Mass Spectrometer used was the QExactive HF mass spectrometer equipped with BioPharma Option (Thermo Fisher Scientific).

ZipChip Method:

Field Strength:	500 V/cm
BGE Type:	Oligos
Injection volume:	1nL
Pressure Assist Start Time:	0.0 min
Analysis time:	5 min

MS Method:

Size	10 mer	20 mer	40 mer
Scan Range (m/z)	800-3000		1000-3000
Resolution	30,000		60,000
Sheath Gas	2		
In source-CID (V)	20	40	80
AGC Target	1.00E+06		
Max IT (ms)	20		
Microscans	3		

Data Acquisition and Processing:

The data was acquired using Thermo Scientific™ Xcalibur4.0 and separations were visualized using Thermo Scientific™ Xcalibur Qual Browser softwares.

RESULTS AND DISCUSSION

Bare Glass Chip Separation Mechanism

The HRB chips have a different chip chemistry compared to the standard polymer coated chips. Unlike the standard polymer coated chips, the separation channels on a HRB chips have a silica surface which results in the channel surfaces having a net negative charge especially at a higher pH. 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). The strong EOF 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 as shown in the schematic in Figure 1.

Figure 1. Schematic showing direction of migration of analytes on a bare glass chip

Characterization of Oligonucleotides

The ZipChip CE/MS provided a rapid and robust method for analysis for oligos. The method employed a BGE with basic pH (~8.5) to ensure a steady EOF in the channels. Either the High-Speed Bare Glass chip (HSB) (p/n 810-0023) or the High-Resolution Bare Glass chip (HRB) (p/n 810-00239) can be used based on user preference. For this work, the HRB chip was used. The HRB chip enabled easy analysis of negatively charged oligonucleotides within 5 minutes. Sharp peaks with gaussian distribution were observed in the electropherograms 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.

Analysis of Unmodified 10, 20 and 40 mer ssRNA Standards

Figure 2 displays base peak electropherograms for 1 uM of solutions of unmodified 10 mer (2a) and 20 mer (2b) and a 2 uM solution of 40 mer (2c) ssRNAs The mass spectra for the three standards are also shown. An insert in the mass spectra shows a zoomed-in view of the $[M+3H]^{3+}$ $[M+4H]^{4+}$ and $[M+5H]^{5+}$ species that

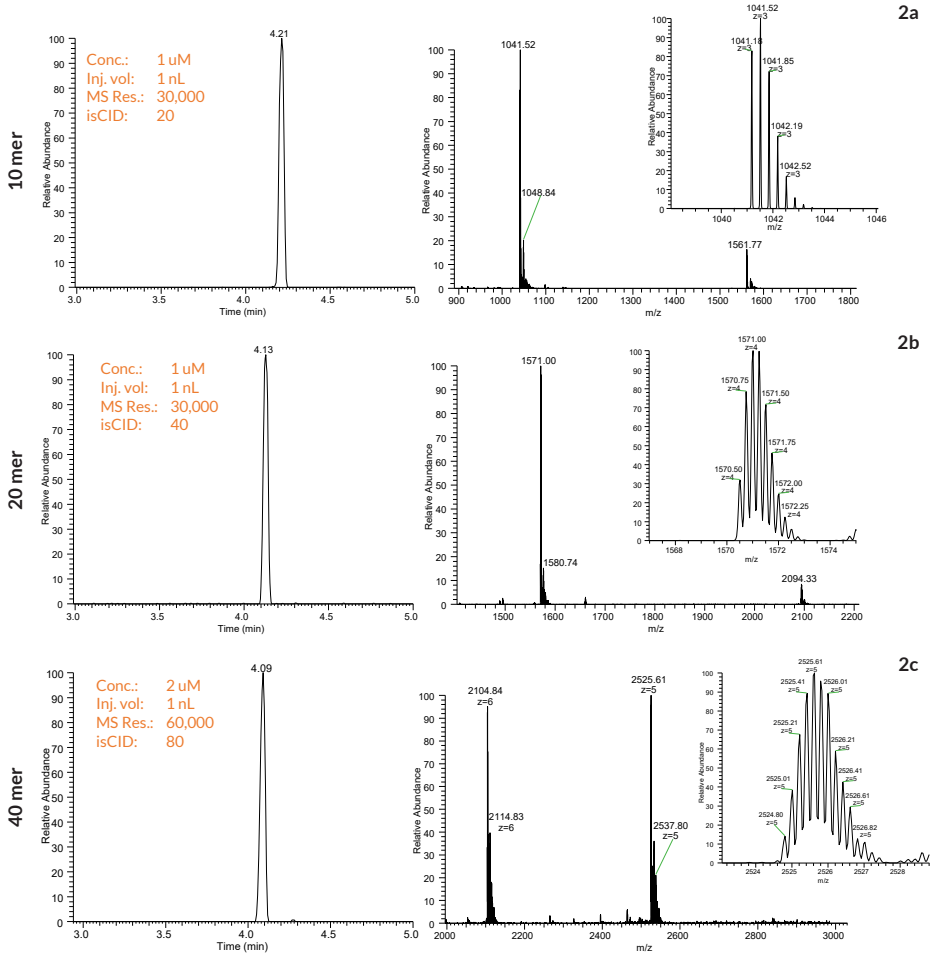

Figure 2. Base Peak Electropherograms for 10 (2a), 20 (2b) and 40 (2c) mer unmodified ssRNA standards (left) along with their raw mass spectra (right). The insert shows a zoomed-in view of the prominent charge states

were detected as the dominant charge states for the 10, 20 and 40 mer ssRNAs respectively. Optimization of certain MS method parameters such as resolution and in-source fragmentation energy was necessary for different sized oligos to obtain optimal peak intensities. The details of the MS method parameters for each sized oligo standard are outlined in the Materials and Methods section. In all three cases, sharp electrophoretic peaks with clean mass spectra were obtained for each standard in less than 5 minutes as show in Figure 2. The protonated adduct was observed as the most prominent adduct in the mass spectrum. Other minor alkali-metal ion adducts such as sodium and potassium were also detected. The level of adduction appeared to be increasing with the size of the oligos.

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. The modified standards had the same sequence and included modifications such as phosphorylation, biotinylation or 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 electropherogram in Figure 3.

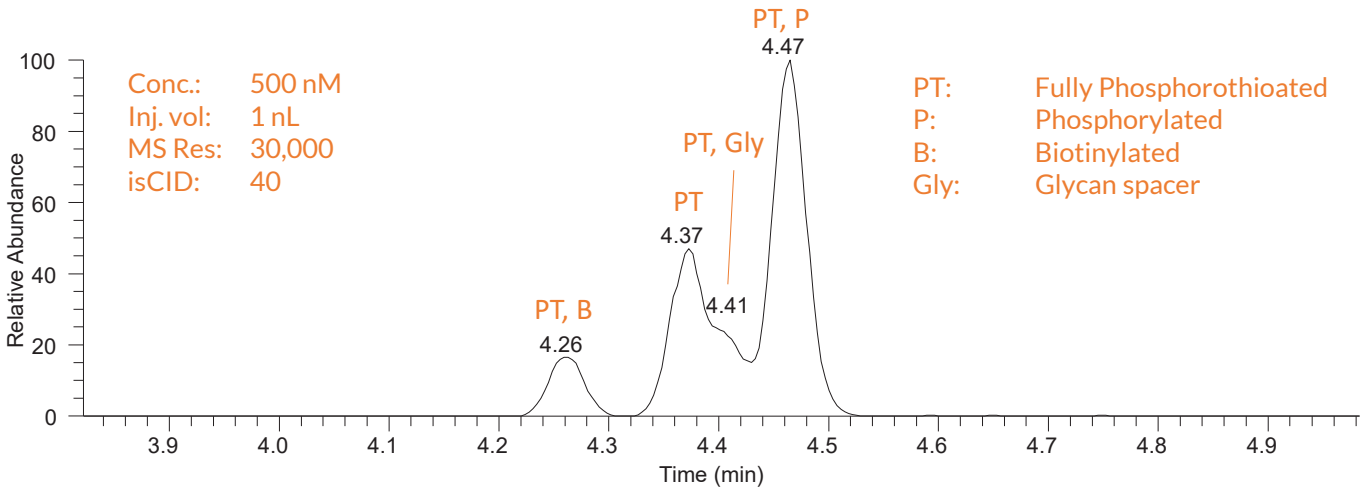

Figure 3. Electropherogram showing separation of biotinylated, glycosylated and phosphorylated 20 mers from the corresponding unmodified 20 mer RNA standard

In a separate experiment, 100 nM of phosphorylated 20 mer ssRNA was spiked into a 1 uM solution of the corresponding non-phosphorylated 20 mer ssRNA standard. Due to the presence of an additional negative charge on the phosphorylated molecule, it was detected as a baseline resolved peak from the non-phosphorylated molecule as shown in the Figure 4a. The resulting mass spectra for the two peaks are also shown in Figure 4b. The order of migration was in accordance with the net charge of the molecules with the molecule with less net negative charge migrating first.

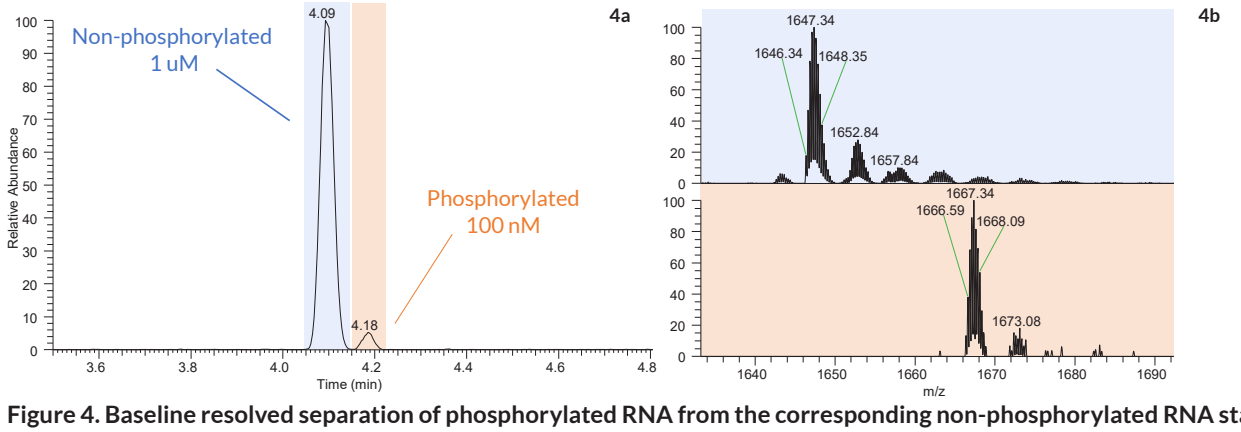

Figure 4. Baseline resolved separation of phosphorylated RNA from the corresponding non-phosphorylated RNA standard

Robustness and Reliability

After demonstrating separation, attention was focused on determining the robustness and reliability of the method. A 1 uM solution of unmodified 20 mer ssRNA was repeatedly analyzed with a total of 375 injections over 3 consecutive days. Three different HRB chips were used for this study with 125 injections per chip. The BGE in the chip wells was refreshed after every 3 injections. A representative plot from one 125 injection sequence is shown in Figure 5. The data demonstrates the high migration time and peak area reproducibility of the method. As shown, the relative standard deviations for peak area and migration time were 14.9% and 1.4% respectively.

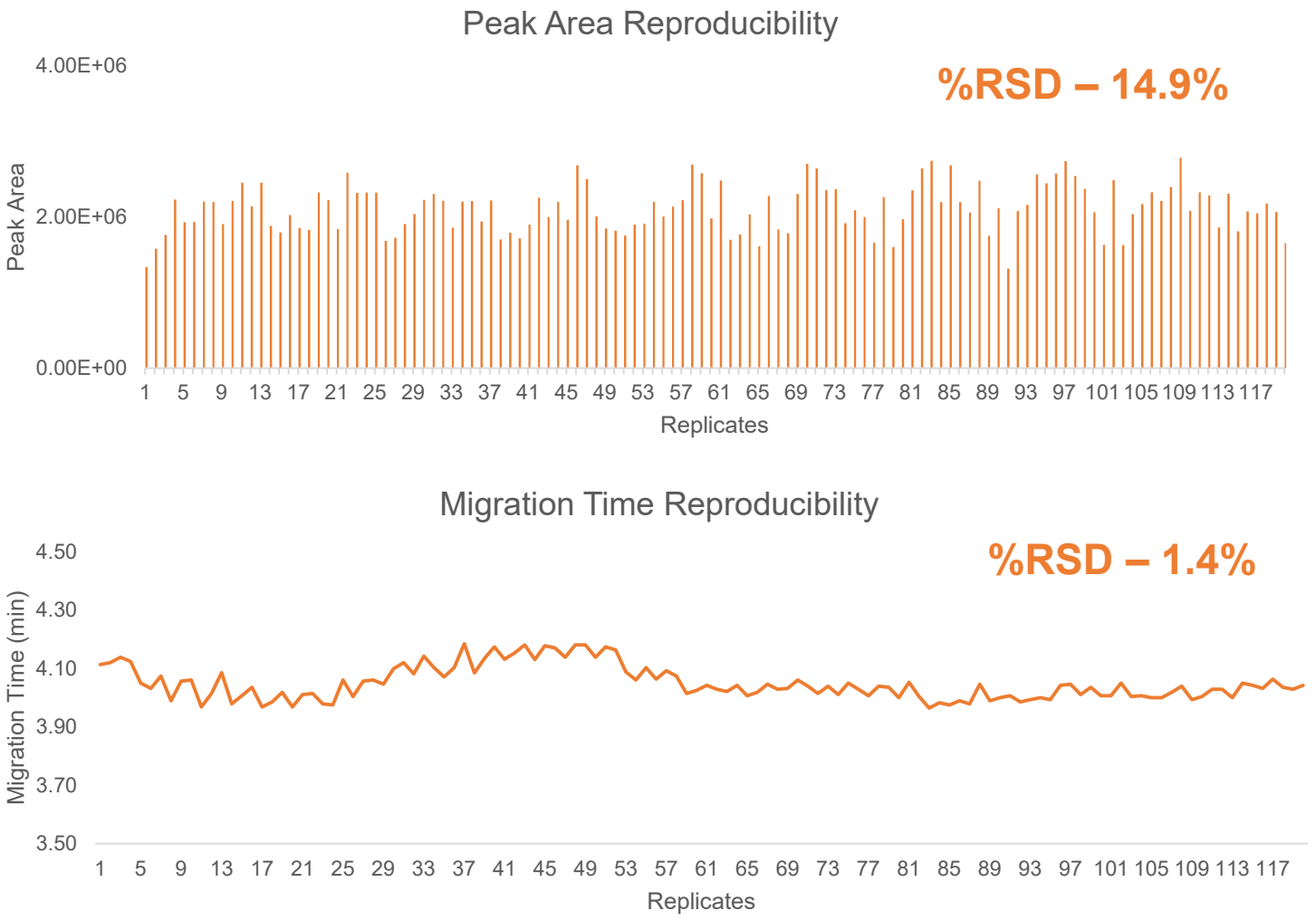

Figure 5. Replicate injections showing peak area (top) and migration time (bottom) reproducibility

Linearity, Dynamic Range and Sensitivity

The sensitivity of the method was tested using the unmodified 20 mer ssRNA standard. The standard was serially diluted from a 500 uM stock solution to concentrations of 10000 nM, 5000 nM, 1000 nM, 500 nM, 100 nM, 50 nM, 10 nM and 5nM using the BGE. Each calibration standard was injected in duplicate. The calibration curve for a 20 mer standard is shown in Figure 6. The method was highly linear within the dynamic range of 5 nM - 10000 nM with an R^2 value of 0.99. The LOD and LOQ were found to be 5 nM and 10 nM respectively demonstrating high sensitivity.

CONCLUSION

The ZipChip CE/MS method 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 and does not require toxic ion-pairing agents, eliminating instrument down time due to

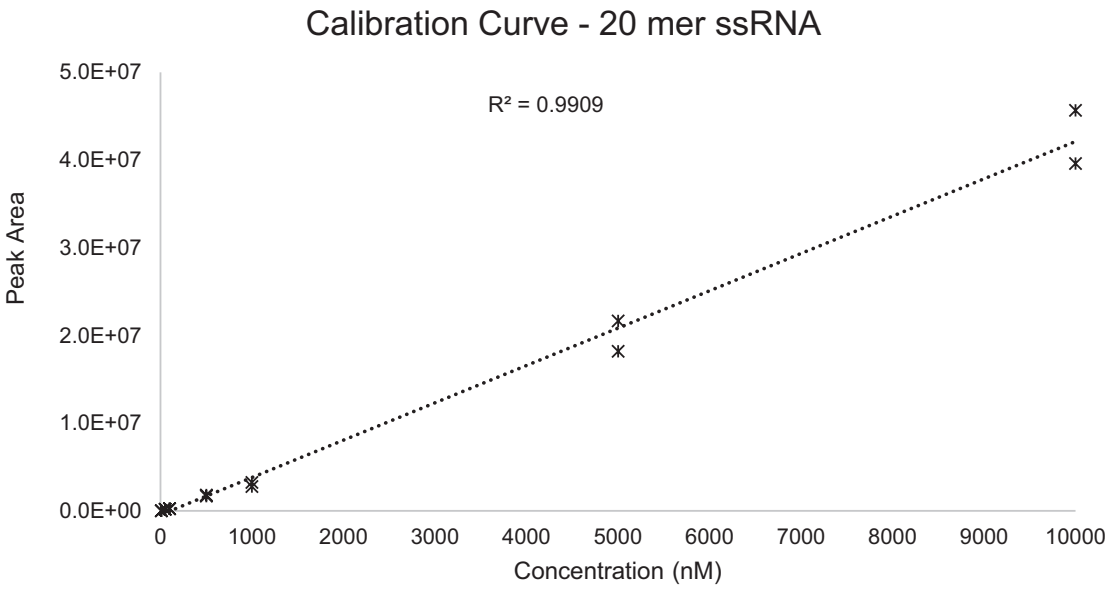

Figure 6. Calibration Curve showing good linearity and dynamic range for the unmodified 20 mer ssRNA standard

contamination and thus, enables fast switching between different applications. The method exhibits linearity over a wide dynamic range, can separate phosphorylated, biotinylated and glycan spacer-containing oligonucleotides from the corresponding unmodified oligonucleotides and shows excellent peak area and migration time reproducibility.

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

The ZipChip oligos kit.

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