Rapid characterization of oligonucleotides and related impurities using microchip CE-MS without ion pairing reagents

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Highlights

- Microchip CE/MS characterization of oligonucleotides with ZipChip/Exploris 240
- Plug and play, easy and clean analysis with ready to use micro chip and Oligos BGE
- No ion pairing reagents, no method development, minimum sample preparation
- Versatile applications: FLP MW, separation of modifications, impurity analysis
- Rapid analysis: monitor of forced degradation products in 1.5 minutes analysis time

Introduction

Reverse Phase LC (RPLC) utilizing ion pairing reagents coupled with MS remains to be the most common approach for oligonucleotides (Oligos) analysis. However, typically a dedicated LC/MS system is required, since the ion-pairing reagents [such as triethylamine (TEA), hexafluoroisopropanol (HFIP)] required for the LC separation contaminate LC/MS system. It is also very demanding for mass spec maintenance. The RPLC separation also requires extensive method development time. In addition, it is highly possible that regulatory agencies require companies to provide assay data orthogonal to RPLC for oligos during IND filing. Orthogonal separation methods such as HILIC suffer from poor column stability and long equilibration time between runs. Herein, we describe a simple & fast method for oligonucleotide characterizations using a Microchip CE/MS platform. This approach involves a chip-based capillary-electrophoresis separation coupled with a high-resolution mass spectrometer (HRMS). It enables efficient analysis of Oligos by accurate mass in positive nanoESI mode.[1] The ready to use Oligos background electrolyte (BGE) solution is free from ion-pairing reagents and makes switching between applications simple and easy. This vendor agnostic protocol requires no method development, can be easily adopted for Oligos characterization workflows such as intact mass analysis, analysis of impurities and degradation products.

Materials & Methods

Samples: All oligonucleotides were purchased as lyophilized standards from Integrated DNA Technologies Inc. (IDT). ***Intact analysis:*** The standards were reconstituted with the Oligos diluent (908 Devices Inc.) to appropriate concentrations prior to analysis. ***Separation of Modified Oligos*:** A mixture of 4 Oligos with 500 nM equal concentrations was used. The 4 Oligos were: a fully phosphorothioated 20 mer RNA, plus 3 other Oligo modifications (phosphorylation, biotinylation, and glycan spacer). ***Dynamic range*:** 20 mer RNA standard was serially diluted from 10uM up to 5 nM using the Oligos diluent (908 Devices). ***Synthetic impurities*:** Oligo with the same sequence and modifications as Nusinersen (10uM in Oligos diluent) was spiked with its n-1, n-2 and n-3 shortmer impurities on 5' and 3' ends at concentration of 1nM. ***Heat Stress Study*:** A 21mer DNA standard (0.1 mg/mL in nuclease-free water) was heated at 80 °C for 1, 2, 4, 6, 8, 12 and 24 hours. Samples were directly loaded on the chip for analysis.

Instrumentation: A ZipChip™ Device (908 Devices Inc.) was used for all analyses. Bare Glass Chip: High Resolution (HRB) or High Speed (HSB) plus the Oligos BGE (908 Devices Inc.) were used. On-chip injection volume was 1 nL. Field strength was 500 V/cm for HRB and 1000V/cm for HSB chip. MS analysis was performed on either a Orbitrap Exploris™ 240 or QExactive HF mass spectrometer (Thermo Fisher), data acquisition mode was ESI+.

Data Processing: The data was visualized using the Qual Browser data analysis software (Thermo Fisher Scientific). Raw data file was parsed and processed in BioPharma Finder 5.0 (Thermo Fisher Scientific) data processing software. Forced degradation data was processed using Skyline (v 21.2).

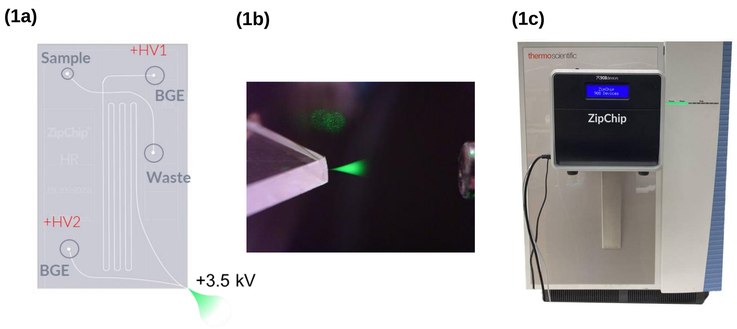

Figure 1. *ZipChip CE/MS system.* ***(1a),*** *Schematic of a ZipChip: microfluidic capillary-zone-electrophoresis + nanoESI spray.* ***(1b),*** *Live monitor of the spray: corner spray illuminated green laser.* ***(1c),*** *ZipChip/Orbitrap Exploris 240 System*

Rapid MW Confirmation of FLP

The ZipChip based CE/MS platform enables rapid analysis for intact oligos. For this work, we analyzed oligos from 10-80 mers with a generic CE separation protocol. The MS parameters were optimized for each molecule: higher in-source CID (isCID) and higher MS resolution were required as the size of the Oligos increases. Figures 2a-2c show the electropherograms for 20, 50 and 80 mer DNA standards, the corresponding mass spectra are shown in Figures 2d-2f, each of the MS spectrum includes a zoomed in spectrum of the dominant charge state. For Oligos analysis with ESI+, only one or two dominant charge states were observed. This greatly simplified post acquisition data deconvolution. For example, for the 20 mer DNA, the dominant charge state was +4 **(Figure 2d)**; while for the 80mer, the dominant state was +9 **(Figure 2f)**.

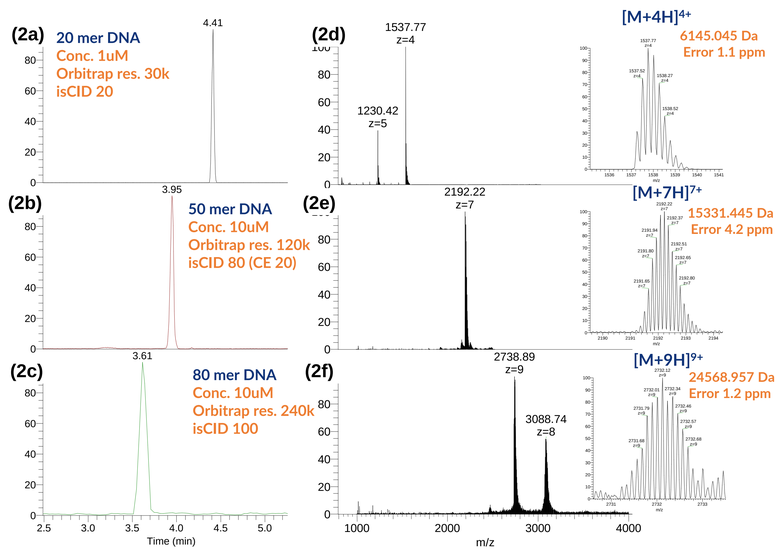

Figure 2. *Extracted Ion Electropherograms of DNAs 20mer, 50mer, 80mer (2a-2c); Raw mass spectra for the corresponding DNA (2d-2f), inserts show M+H adduct in the dominant charge state for each of the Oligos respectively.*

To further assess the CE/MS method, we analyzed a mixture of modified and unmodified RNAs, the modifications included phosphorylation, biotinylation and a glycan spacer consisting of 1',2'-dideoxyribose on the same RNA sequence. Result is shown in **Figure 3**: the phosphorylation and biotin modified RNAs were baseline resolved from the unmodified RNA (4.37 min), the dideoxyribose modified RNA peak was partially resolved from the main peak.

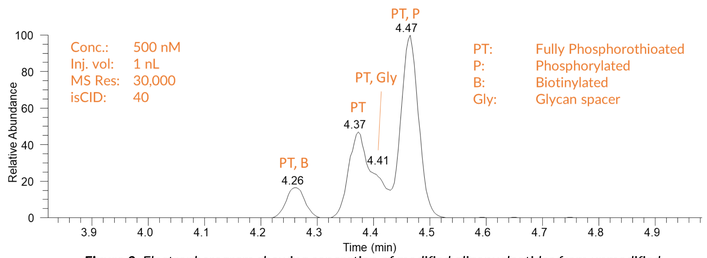

Figure 3. *Electropherogram showing separation of modified oligonucleotides from unmodified.*

Quantification Evaluations for FLP Analysis

The ZipChip CE/MS Oligos method was evaluated for its quantification limits (linear dynamic range and sensitivity) using an unmodified 20 mer ssRNA. Sample for each concentration was injected in duplicates. **Figure 4** shows a linear curve with 5 nM - 10000 nM dynamic range (R^2 0.99), LOD was 5 nM, LOQ was 10 nM.

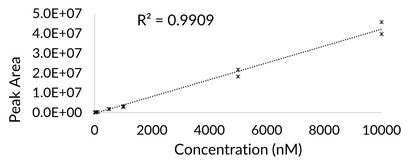

Figure 4. *ZipChip CE/MS Calibration curve and linearity for 20mer ssRNA standard*

Impurity Analysis of FLP

Shortmer Analysis: **Figure 5** shows the result of truncated impurities analyzed by the ZipChip CE/MS method. All 6 shortmer impurities (viz. 5' n-1, 5' n-2, 5' n-3, 3' n-1, 3' n-2 and 3' n-3), spiked at 0.1% of FLP, were easily detected by HRMS within a short analysis time, even though they migrate closely to the FLP. With ESI+, less charge states obtained, simplified the data analysis.

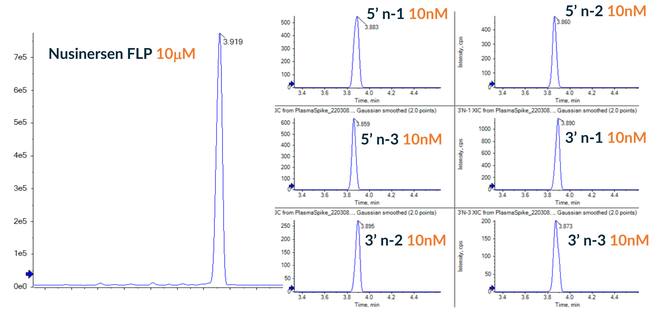

Figure 5. *Extracted ion electropherograms showing the FLP (10 µM) and 0.1% impurities (10 nM)*

Forced Degradation Studies: Heat stressed samples aliquoted at regular time points were analyzed twice, once with HSB chip and once with HRB chip. The analysis time was reduced to 1.5 min on the HSB chip (**Figure 6a & 6c**) compared to 5 min on the HRB chip (**Figure 6b & 6d**). Despite shorter analysis times, the peak profiles and trends in the data observed for HSB chip were similar to that of that HRB chip resulting in significant time savings for analyzing large cohorts of samples without compromising the data quality. As shown in **Figures 6a- 6d** the peak profiles for heat stressed samples analyzed on high-speed and high-resolution chips were identical. The 21 mer FLP appeared to be significantly degraded after 24h and a large number of degradation products were observed. There was a marked decrease in the abundance for the FLP during the 24 h period (**Figures 6a, 6c & 6e**). Progressively larger number of degradants were detected at longer time points. Truncated degradation products containing phosphorylation modification showed a large increase with time. Examples shown here include phosphorylated 5' n-15 (**Figure 6e**) and a phosphorylated 5' n-18 species (**Figure 6f**).

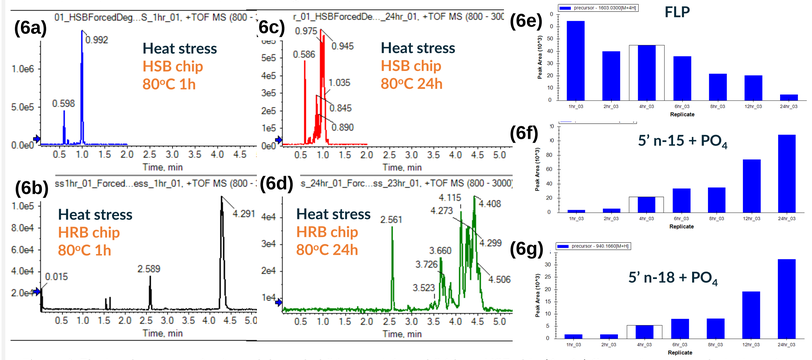

Figure 6. *Electropherograms for forced degraded 21 mer at 1, and 24 h on HSB chip (6a-6c) Comparison of analysis times for the same 24h forced degraded sample on HSB (6c) and HRB (6d); Abundances of the FLP, phosphorylated 5' n-15 and phosphorylated n-18 impurities over a 24 h time period (6e-6g).*

Conclusions & Future Work

- Oligos (20-80 mer) easily analyzed with the Microchip CE/MS without method development
- The CE/MS method showed excellent sensitivity with wide dynamic range
- Oligo modifications (induced separate charge states or hydrodynamic radii) were resolved form the corresponding unmodified oligo in <5 min
- The method can be adopted for analysis of impurities from the oligo FLP
- Heated stress samples were analyzed on HSB chip in less than 1.5 minutes
- Future work will be focused on assessing the performance of the HSB chip for qualitative and quantitative analysis of synthetic oligonucleotides.

References

1. Application Note 9.5 "Rapid characterization of oligonucleotides using microfluidic capillary electrophoresis - mass spectrometry by ZipChip. 908 Devices

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The technologies discussed in this poster are the subject of one or more granted/pending patents. www.908devices.com/patents/