Microchip CE-MS Analysis of Nucleic Acids: From Characterization of Synthetic Oligonucleotides to Sequence Mapping of RNAs

Catherine Rawlins[1] • **Scott Mellors**[2] • **Adi Kulkarni**[1] • **Kate Yu**[1]
[1]908 Devices Inc., Boston, MA • [2]908 Devices Inc., Morrisville, NC

REPLIGEN

Repligen Corporation now owns the life sciences PAT product portfolio of 908 Devices Inc. Please contact Repligen for further inquiries.

Highlights

- The PATsmart™ ZipChip® System can perform versatile applications from AXPs, to synthetic oligos, to RNAs
- The analysis is rapid and simple with application specific and ready to use consumables (Chip & BGE)
- Requires no Ion-Pairing (IP) reagents, easy method development, and minimum sample preparation

Introduction

ZipChip is a microchip-based CE-MS platform that performs rapid and efficient analysis applicable to versatile fields such as biopharma, clinical diagnostics, gene and cell therapies, and multi-omics. Here we focus on the rapid analysis of nucleic acids, with special focus on oligonucleotides.

In comparison, reverse phase LC-MS method for oligos is known for its contamination due to ion-pairing reagents, and the HILIC LC-MS method still faces challenges such as long equilibration times and poor column stability. The ZipChip serves as an ideal alternative and orthogonal approach, enabling rapid, efficient, and ion-pairing reagent free analysis for oligonucleotides and mRNA. The workflow requires minimal method development, is vendor agnostic, and allows for easy switching among different applications with no extra time needed for clean system contamination or sample carryover.

We present three application examples: 1) the analysis of AXPs (Adenosine, AMP, ADP, and ATP) spiked in human plasma extract, 2) the analysis of synthetic oligos with different sizes and modifications, and 3) bottom-up oligo mapping for a Rnase T1 digest of HIV-1 5'-UTR (untranslated region) RNA (UTR). Overall, the ZipChip workflow for oligonucleotide applications demonstrates excellent resolution and sensitivity with speed and simplicity.

Materials and Methods

Samples

Adenosine (Ade), adenosine 5'-monophosphate monohydrate (AMP), adenosine 5'-diphosphate (ADP), and adenosine 5'triphosphate disodium salt hydrate (ATP) were purchased from MilliporeSigma. Normal human plasma (3.2% NaCit gender pooled 0.2 µm filtered) was purchased from BioIVT.

All oligonucleotides were purchased as lyophilized standards from Integrated DNA Technologies Inc. (IDT). UTR was produced in collaborator's lab at University of Connecticut by *in vitro* transcription.

For additional details on sample preparation for each application, please refer to the references listed at the end of this poster.

Instrumentation

A ZipChip Device (Repligen) was used as the front-end Microchip CE for all analyses. The consumables were: High Resolution Bare Glass Chip (HRB), High Speed Bare Glass Chip (HSB), the Oligonucleotide Background Electrolyte (BGE) (Repligen). Injection volume was 1 nL for AXPs, 1.5 nL for oligos, and 0.5 nL for bottom-up analysis of UTR. CE field strength was 500 V/cm for the AXPs and bottom-up on an HRB and 750 V/cm for oligos on an HSB chip.

The Nano-ESI MS analysis was performed on the Q Exactive HF for AXPs, the Orbitrap Exploris 240 (Thermo Fisher Scientific) for the oligos, and the timsTOF Pro (Bruker) for the bottom-up oligo mapping of UTR.

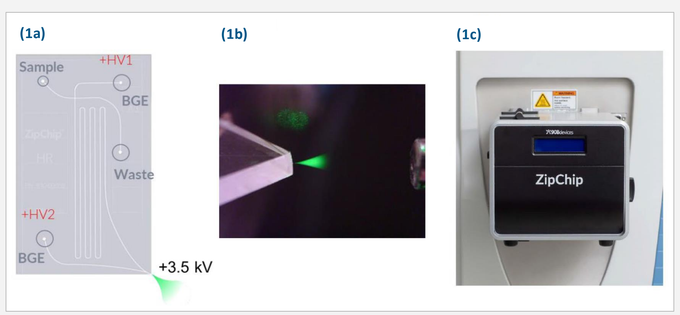


Figure 1. ZipChip CE/MS System. **(1a)** Schematic of a ZipChip: microfluidic capillary-zone-electrophoresis + nanoESI spray. **(1b)** Live monitor of the corner spray illuminated with a green laser. **(1c)** ZipChip interface coupled to high resolution mass spectrometer.

1. Quantitative Analysis of Adenosine, AMP, ADP and ATP from Plasma

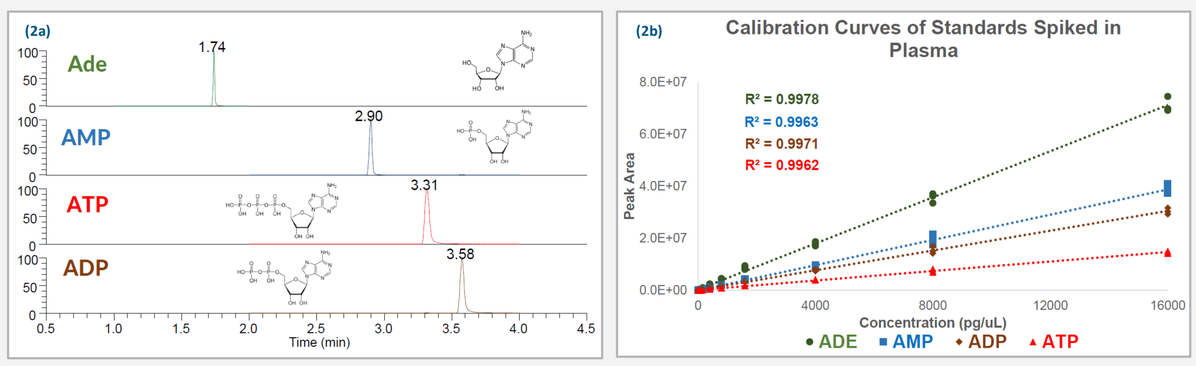
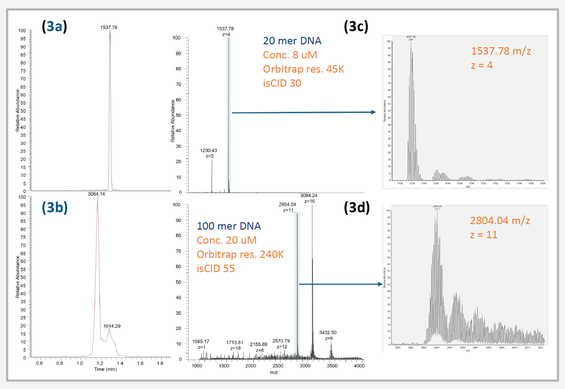


Figure 2. Extracted ion electropherograms from CE-MS analysis for a standard mixture of Ade, AMP, ATP and ADP at 500 pg/µL showing baseline resolved separation of all analytes **(2a)**, and correspondence calibration curves of for each of the standards spiked in human plasma **(2b)**.

The ZipChip CE-MS analysis for AXPs was simple, rapid with no method development required. The separation was initially tested by injecting a mixture of the four analytes at 500 pg/µL onto the ZipChip-MS system, which resulted with narrow and symmetrical electrophoretic peaks, migrating in <4 minutes (Fig. 2a). Further analysis with the four analytes spiked into human plasma demonstrated quantification with calibration curves (Fig. 2b) covering >4 orders of magnitude of dynamic range.

2. Characterization of Synthetic Oligonucleotides



The ZipChip-based CE-MS platform enables rapid analysis for intact oligos. In this example, we analyzed oligos from 10–100mers with the generic CE separation protocol. The MS parameters were specifically optimized for different oligos. As the size of the oligo increases, we increased in-source CID (isCID) energy and the MS resolution in order to achieve isotopic resolution. Figs. 3a & 3b show the electropherograms for 20mer and 100mer DNA standards, with the corresponding mass spectra shown in Figs. 3c & 3d.

Each of the MS spectra includes a zoomed in spectrum of the dominant charge state. With MS data acquisition in positive ESI mode, fewer charge states were observed which simplifies post acquisition data deconvolution. As we analyze larger oligos, the observed adducts do increase; however, the protonated species remains the major peak with oligos up to 100mer (Fig. 3d).

Figure 3. Extracted Ion Electropherograms and raw mass spectra of 20mer and 100mer oligos with inserts showing M+H adduct in the largest charge state for each oligo.

We analyzed a mixture of modified & unmodified oligos, with modifications including phosphorylation, biotinylation, and a glycan spacer consisting of 1',2'-dideoxyribose on the same RNA based oligo sequence. As shown in Figure 4, 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.

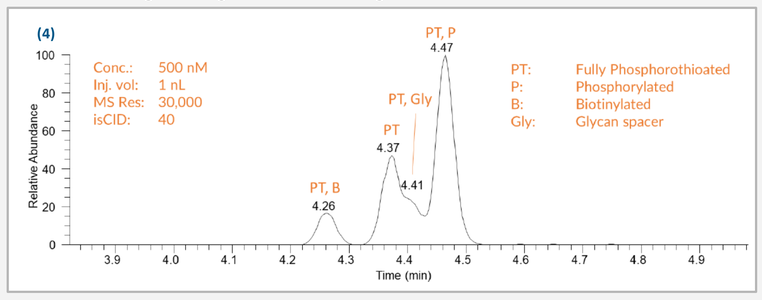


Figure 4. Electropherogram showing separation of modified oligonucleotides from unmodified.

3. Bottom-Up Oligo Mapping for RNAs

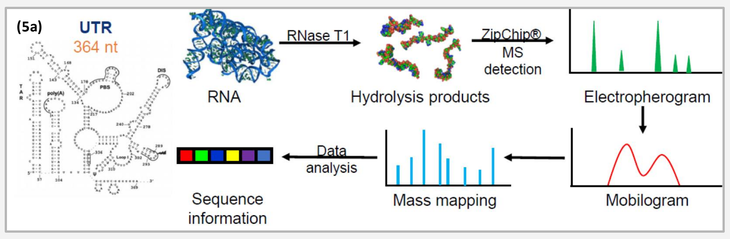


Figure 5. Protocol for digestion and analysis workflow **(5a)**; Bottom-up analysis of UTR on ZipChip on a timsTOF **(5b)**

The mass mapping of UTR, identifying 551 digestion products with an average size of 14.9 nucleotides. In Figure 5b, the annotated MS spectra peaks identified specific digestion products, such as the [1:10] 3+ peak representing a hydrolytic product consisting of nucleotides from the 1st to 10th positions with a +3 charge state. Complete sequence coverage was achieved in an analysis less than 4 minutes.

Conclusions

- The ZipChip platform enables a simple and rapid workflow for the analysis of nucleic acids with no ion-pairing reagents needed.
- Energy metabolites such as Ade, AMP, ADP, and ATP were baseline resolved in less than 4 minutes with >4 orders of magnitude of dynamic range.
- Synthetic oligos (20–100mer) were rapidly analyzed with modifications and were resolved form the corresponding unmodified oligo in <5 min.
- HIV-15'-UTR digested with Rnase T1 was analyzed with 100% sequence coverage in less than 4 minutes.
- The Oligonucleotide BGE kit and the method development is continuing to improve for large oligonucleotides.

References

1. Application Note 9.5 "Rapid characterization of oligonucleotides using microfluidic capillary electrophoresis-mass spectrometry by ZipChip". Repligen
2. "Determination of ATP, ADP, AMP, and Adenosine Levels by Microchip Capillary Electrophoresis Coupled with High Resolution Mass Spectrometry" (Poster); ASMS June 2023; Houston, TX, USA
3. "Rapid characterization of oligonucleotides and related impurities using microchip CE-MS without ion pairing reagents" (Poster); ASMS June 2022; Minneapolis, MN, USA
4. "Continuing the investigation of microchip capillary electrophoresis coupled with mass spectrometry in the bottom-up characterization of progressively larger RNAs" (Poster); ASMS, June 2023; Houston, TX, USA.

Acknowledgments

The authors would like to express their sincere gratitude for the contributions of the following collaborators: Robert Ross and Min Du from ThermoFisher Scientific, Daniele Rollo and Daniel Fabris from the University of Connecticut, and Kevin Kun Guo from Avice Labs.

Learn more at **repligen.com/zipchip**