Bottom-up analysis of progressively larger RNAs by CZE-MS and CZE-MS/MS

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Objective and Highlights

The **objective** of this project was to explore the merits of capillary zone electrophoresis (CZE) as a potential alternative to liquid chromatography (LC), as the front-end separation of choice for analyzing progressively larger RNAs with bottom-up approach.

- The **model samples** consisted of synthetic DNA standards (a 17mer and a 20mer) and RNA substrates tRNA^{Phe} (75 nt) and 18S rRNA (1,869 nt). The constructs were either obtained from commercial sources, produced in house (*in vitro* transcription), or isolated from cell lysates (size exclusion chromatography). When necessary, the samples were extensively desalted by solvent exchange in Amicon (Millipore, Burlington, MA) with 0.5 mL centrifugal filters (3K MWCO).
- The **experimental approach** involved 1) direct analysis of DNA standards; 2) RNA digestion mixtures, which were obtained by treating tRNA^{Phe} and 18S rRNA with RNase T1 (Sigma-Aldrich). The digestion conditions were adjusted to favor the formation of hydrolysis products that contain nucleotides 20-30 nucleotides (nt), which are readily amenable to MS and MS/MS analysis.
- The **characterization** of hydrolytic products was carried out on a microchip CE device – ZipChip (908 Devices), in either direct infusion mode or in CZE separation mode. The ZipChip allows nanoESI spray at the corner of the chip, which is directly positioned in front of the Mass Spec. The Mass Spec used was either a Bruker 7T solarix XR FT-ICR, or a timsTOF Pro. The analysis was carried on by ESI+ mode.
- The **interpretation** of the MS and MS/MS data was carried out with the aid of the RiboDynamics SeqRead algorithms integrated in the Bruker OligoQuest software.

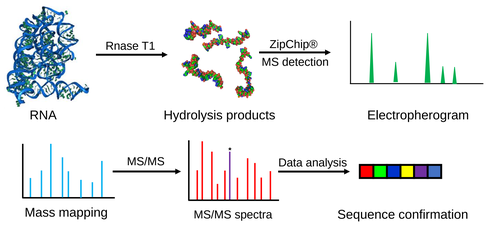
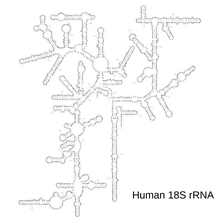


Figure 1. Process describing bottom-up analysis of RNAs by CE-MS.

Challenges of Bottom-Up Analysis for Large RNAs



The 1,869 nt human 18S rRNA is predicted to provide a total of 525 products under limited digestion conditions:

rU-1:rG-23
rU-21:rG-47
rG-95:rG-114
rG-156:rG-183
rG-269:rG-288
rA-447:rG-471
rC-636:rG-659
…

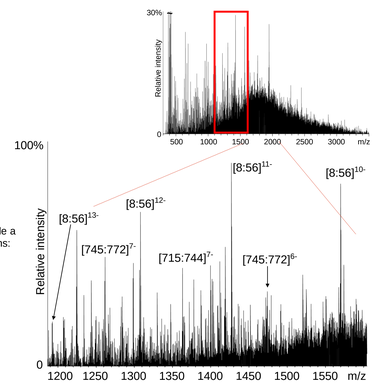


Figure 2. Bottom-up analysis of an 18S rRNA digestion mixture without front-end separation.

- Complex mixtures of oligonucleotides generated by enzymatic digestion.
- Major bottleneck for complicated data deconvolution as a result of the analysis.
- **Pain can be alleviated by utilizing of a fast front-end separation, which can greatly expedite the sought-after assignment of the entire digestion mixture, facilitate the attainment of sequence information.**

Microchip CZE by ZipChip

❖ **Merits of CZE performed on a chip**

- Rapid and simple analysis (~5 min), no method development
- No ion pairing reagents
- Low sample consumption (~1 nL per injection)
- High sensitivity (~ 10 amol sample consumption)

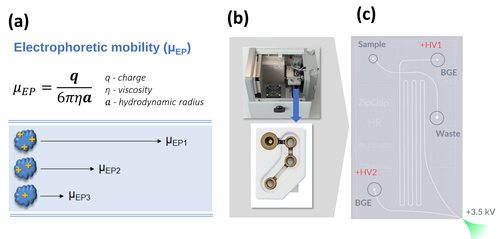


Figure 3. Factors affecting electrophoretic mobility of ions in CZE (a); ZipChip interface and microfluidic device (b); schematic of the microfluidic device (c).

❖ **Analytical protocol**

1. A small sample plug present in Background Electrolytes (BGE) is pressure-injected into the ZipChip from the sample well.
2. Voltage is applied across the separation channel to drive sample migration.
3. Different analytes are separated according to charge and size.
4. The chip generate nanoESI spray. Its electrophoretic setup requires performing electrospray analysis in positive ion mode.

Separation capabilities

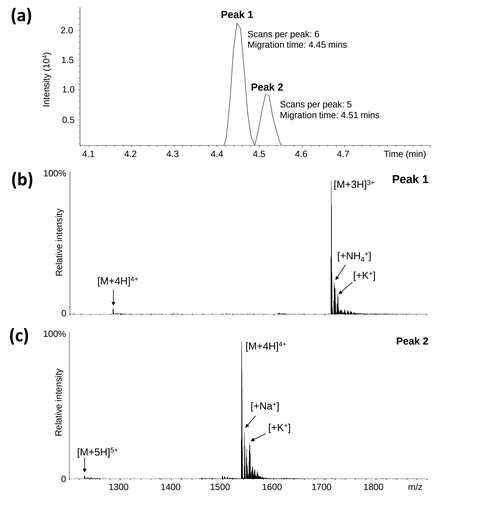
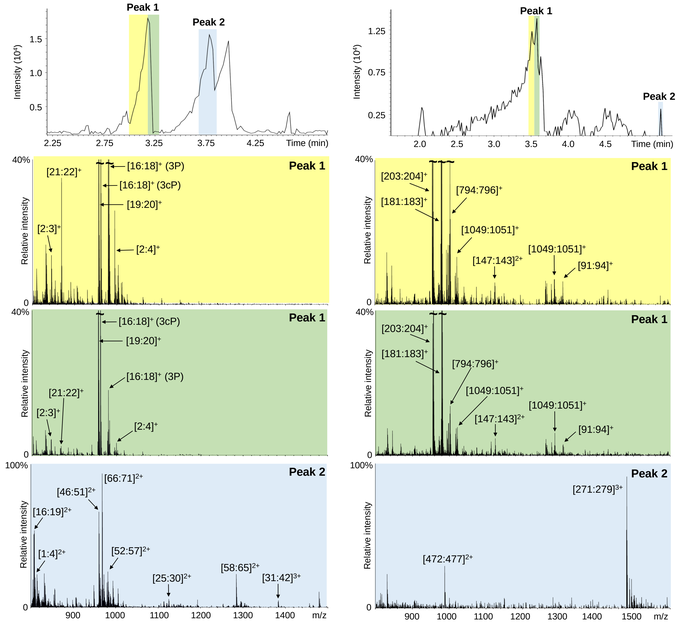
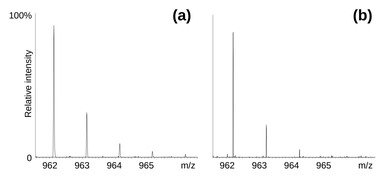


Figure 4. ZipChip separation of 17 mer and 20 mer DNA standards (a); raw mass spectrum of 17 mer (b) and 20 mer (c).

Analyze Complex Digestion Mixtures of RNA)

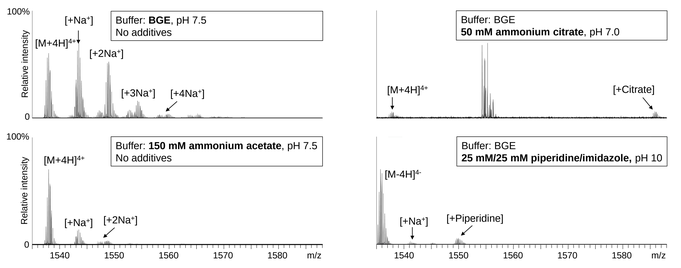


- **Figure 5:** Electropherograms (top panels) and representative mass spectra of tRNA^{Phe} (left column) and 18S rRNA (right column) digests. All data were acquired on timsTOF. 55 products out of 190 predicted hydrolytic product were detected for tRNA^{Phe}, whereas 290 out of 525 for 18S rRNA.



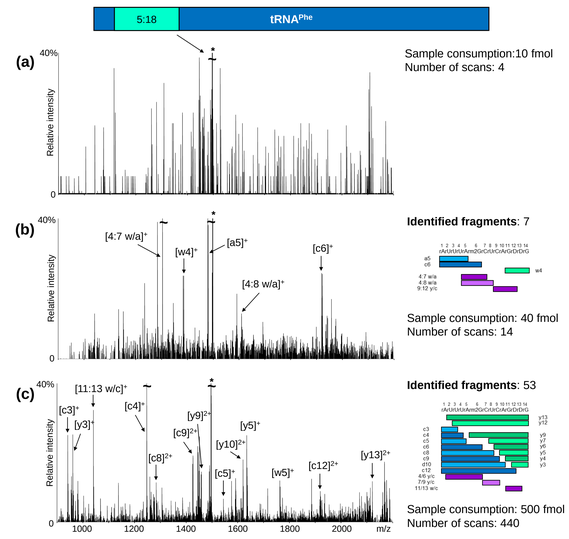
- **Figure 6:** The performance of the mass analyzer can further affect the quality of the acquired data. Notice the ~40,000 vs. 70,000 resolution afforded by a) timsTOF vs b) FT-ICR determinations on the analysis of the [16:18](3P) digestion product. Both spectra were acquired in separation mode with ZipChip.

Optimizing MS analysis with BGE



- **Figure 7:** Static nanoESI mass spectra of the DNA 20mer standard collected on FT-ICR in either positive or negative ion mode. Only one charge state is shown to highlight the effects of the various conditions on the extent of salt adduction. The buffers and additives explored here were selected for their reported ability to reduce cation adduction.

MS/MS by Infusion and CZE Separation



- **Figure 8:** MS/MS spectra acquired on timsTOF for a representative digestion product of tRNA^{Phe}. The data acquired in separation mode (panel a) utilized only 10 fmol of sample, whereas those acquired by static nanoESI employed 500 fmol. The disparity of spectral quality and information afforded by the two experiments is explained by the difference in the number of ions that underwent gas-phase activation. The statistics shown were determined with the support of the SeqRead algorithms and OligoQuest software.

Conclusions and future directions

- Capillary zone electrophoresis performed on a chip proved to be an effective **alternative to liquid chromatography** as a front-end separation system to analyze both pure samples and complex digestion mixtures of nucleic acids.
- MS/MS experiments performed in **positive ion mode** verified the sequence information of RNA digestion products.
- We are currently investigating the effect of additional chelating agents and other experimental conditions (including concentration of the sample, pH, and ionic strength) on the separation performances and the intensity of the signals observed in MS/MS.
- We are exploring **trapped ion mobility** as a potential aid in the separation provided by CZE and in assessing the presence of isomers or different conformations assumed by certain oligonucleotides.

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Acknowledgments

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