Rapid Analysis of Oligonucleotides by High-Resolution Accurate Mass CE-MS

Dr. Daniel M. Waldera-Lupa | Laboratory Manager
daniel.waldera-lupa@protagene.com

20th September 2022
Oligonucleotides

- Synthesized short DNA or RNA molecules (single or double stranded)
- Wide range of applications in molecular biology, biotechnology and therapy
- Oligonucleotides usually contain 10 to 30 nucleotides
- Classification of oligonucleotides:
  - Antisense oligonucleotides (ASOs)
  - Short interfering RNA (siRNA)
  - MicroRNA (miRNA)
  - Aptamers
  - CpG oligonucleotides

Oligonucleotide Therapeutics

• Direct therapeutics against a variety of diseases:
  – Oncology, infectious diseases, metabolic disorders, genetic disorders, etc.

• Fifteen oligonucleotide therapeutics have been approved since 1998
  – The majority in the last five years
  – Many more are in development

• ASOs
  – ~18-30 nt, single stranded
  – Degradation of mRNA, suppression of transcription, inhibition of translation

• siRNA
  – ~19-23 nt, RNA duplex
  – Inhibition of translation, degradation of mRNA
Quality Control

- Confirmation of oligonucleotide mass and sequence
- Quantification of purity level
- Product- and process-related impurities determination and quantification:
  - Addition sequences (longmers, n+1)
  - Deletion sequences (shortmers, n-1, most common)
  - Phosphodiester analogs
  - Depurinated sequences
  - Modifications

Capillary Electrophoresis using ZipChip®

- Capillary Zone Electrophoresis (CZE) separation
- Electrophoretic mobility (function of the charge-to-size ratio)
- Separation on a High-Resolution Bare Glass chip
- No ion-pairing agents required
- Low sample amounts needed (injection of 1 nL)
- Fast analysis (<10 min)

https://www.thermofisher.com/order/catalog/product/de/de/00950-01-00494
The ZipChip®

- Single piece of glass
- Microfluidic channels by photolithography and wet chemical etching
- Three major functional elements:
  - sample injection
  - electrophoretic separation
  - electrospray ionization

Tech Note 1.0 ZiChip What they are and how they work. 908devices
CE Separation of Oligonucleotides using ZipChip®

- Following the sample injection, voltage is applied to the BGE channels.
- These voltages dictate the electrical field strength for the CE separation and the ESI voltage.
- Analytes migrate down the separation channel in the electric field toward the ESI emitter.
- Negatively charged oligonucleotides are carried to emitter with EOF.
Capillary Electrophoresis Mass Spectrometry (CE-MS)

- Direct coupling to the mass spectrometer
  - Positive ESI mode
  - Stable nano-spray with high sensitivity
  - High-resolution accurate mass (HRAM) (60k resolution)
- Data evaluation with BYOS® Oligos
  - Identification (via MS/MS)
  - Quantification (peak integration)

https://www.thermofisher.com/order/catalog/product/de/de/00950-01-00494
CE-MS of Oligonucleotides
Method Development
Separation of Oligonucleotides by CE-MS

- DNA oligo length standard 10/60 Ladder (ssDNA, Integrated DNA Technologies)
- HRAM CE-MS analysis in a single-shot experiment
- The migration order ranged from low to high molecular weight, with the exception of the 10mer
- Correlating with the molarity, the 10mer showed the highest intensity

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Molecular Weight [Da]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mer</td>
<td>3,043</td>
</tr>
<tr>
<td>15mer</td>
<td>4,634</td>
</tr>
<tr>
<td>20mer</td>
<td>6,117</td>
</tr>
<tr>
<td>25mer</td>
<td>7,642</td>
</tr>
<tr>
<td>30mer</td>
<td>9,191</td>
</tr>
<tr>
<td>40mer</td>
<td>12,274</td>
</tr>
<tr>
<td>50mer</td>
<td>15,379</td>
</tr>
<tr>
<td>60mer</td>
<td>18,493</td>
</tr>
</tbody>
</table>
High-Resolution Mass Spectra

- One to two charge states per oligonucleotide
- \([\text{M}+3\text{H}]^{3+}\) to \([\text{M}+7\text{H}]^{7+}\) species were the dominant charge states
- Isotope-resolved mass spectra
- Monoisotopic masses
- Alkali-metal ion adducts such as sodium and potassium were detected
Benefits of HRAM-MS

- HRAM-MS led to high specificity and a low mass deviation
- Unambiguous assignment of MW based on monoisotopic mass
- Quantification with high sensitivity by extracted ion electropherogram (EIE)
  - Most abundant charge state
  - Highest abundant isotope
Quantification of Oligonucleotides

- Dilution series from 0.02 pg (1.1-6.6 nM) to 20 pg (1.1-6.6 µM)
- The quantification was linear over several orders of magnitude
Limit of Quantification

- LOQ within the low nM range
- Not reached for smaller oligonucleotides (10-30mer)
- CE-MS showed a high sensitivity

<table>
<thead>
<tr>
<th>Oligonucleotide Length</th>
<th>Molecular Weight [Da]</th>
<th>Absolute amount [pg]</th>
<th>Concentration [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3,043</td>
<td>&lt;0.02</td>
<td>&lt;6.49</td>
</tr>
<tr>
<td>15</td>
<td>4,634</td>
<td>&lt;0.02</td>
<td>&lt;4.32</td>
</tr>
<tr>
<td>20</td>
<td>6,117</td>
<td>&lt;0.02</td>
<td>&lt;3.27</td>
</tr>
<tr>
<td>25</td>
<td>7,642</td>
<td>&lt;0.02</td>
<td>&lt;2.62</td>
</tr>
<tr>
<td>30</td>
<td>9,191</td>
<td>&lt;0.02</td>
<td>&lt;2.12</td>
</tr>
<tr>
<td>40</td>
<td>12,274</td>
<td>0.05</td>
<td>4.07</td>
</tr>
<tr>
<td>50</td>
<td>15,379</td>
<td>0.20</td>
<td>13.00</td>
</tr>
</tbody>
</table>
CE-MS Analysis of Synthesized Oligonucleotides

- Two individually synthesized 10mers (not purified) were measured
- Data evaluation with BYOS® Oligos
- The 10mers showed baseline separation
- Multiple impurities were detected

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Molecular Weight [Da]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mer,A</td>
<td>CAAAGCGACG</td>
<td>3,046</td>
</tr>
<tr>
<td>10mer,B</td>
<td>GATCCAGACC</td>
<td>2,997</td>
</tr>
</tbody>
</table>

Total Ion Electropherogram
Sequence Confirmation by MS/MS

- The oligonucleotides were identified based on precursor mass and high quality MS/MS spectra
- High ion series coverage was achieved
Identification and Quantification of Impurities

- Impurities present due to low purification grade
- Identification by MS/MS spectra
- Quantification based on MS level
- CE-MS allows for identification and quantification of impurities
CE-MS Analysis of Semi-Complex Mixtures

- Five oligonucleotides of same length and similar mass were analyzed
- A good separation was achieved
- Isobaric compounds can be separated

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Molecular Weight [Da]</th>
<th>Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mer, A</td>
<td>CAAAGCGACG</td>
<td>3,046</td>
<td>4</td>
</tr>
<tr>
<td>10mer, A shuffled</td>
<td>GCCACGAAGA</td>
<td>3,046</td>
<td>5</td>
</tr>
<tr>
<td>10mer+methyl, A</td>
<td>GAC [5MedC] AATCCG</td>
<td>3,012</td>
<td>1</td>
</tr>
<tr>
<td>10mer, B</td>
<td>GATCCAGACC</td>
<td>2,997</td>
<td>3</td>
</tr>
<tr>
<td>10mer,rev. comp., B</td>
<td>GGTCTGGATC</td>
<td>3,059</td>
<td>2</td>
</tr>
</tbody>
</table>

Total Ion Electropherogram
Sequence Confirmation by MS/MS

MS/MS

MS

MS/MS

MS/MS

ProtaGene
Sequencing of Longer Oligonucleotides

Total Ion Electropherogram

MS

MS/MS

[Graphs and diagrams showing sequencing results]
Conclusions

• CE-MS is a suitable tool for rapid analysis of oligonucleotides in quality control:
  – CZE separation using a ZipChip® device
  – Fast analysis (<10 min)
  – Low sample amounts needed
  – No ion-pairing agents required
  – Isobaric compounds can be separated
  – High-resolution accurate mass leads to high specificity
  – The quantification is linear over several orders of magnitude
  – High sensitivity (LOQ in the low nM range)

• Identification is easily achievable with BYOS® Oligos:
  – Molecular weight verification with accurate mass
  – Sequence verification via high quality MS/MS spectra
  – Fast and easy impurity analysis including identification and quantification
Acknowledgements

- Anne Oltmanns
- Melissa Motzkau
- Yvonne Jasper
- Heiner Falkenberg
- Simon Krabbe
- Silvio Keckes
- Kate Yu
- Gary Paul
- Sabine Springer
- Lucy Fernandes