High-throughput LC/MS characterization of mRNA therapeutics using a fast DDA method on the Orbitrap Astral MS

Tabiwang Arrey[1]; Angela Criscuolo[1]; Matthew James Garland[1], Bernd Hagedorn[1], Keeley Murphy[2]; Eugen Damoc[1], [1]Thermo Fisher Scientific, Bremen, Germany; [2]Thermo Fisher Scientific, San Jose, CA

Abstract

Purpose: develop a workflow covering sample preparation, fast LC-MS analysis, and data processing to characterize mRNA therapeutics.

Methods: Partial mRNA digestion was performed using immobilized RNase T1 on magnetic beads for 5, 10 and 15 minutes. The samples were then separated using a Thermo Scientific™ DNAPac RP ™ and LC-MS/MS analyses were performed in negative ion mode on a Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer. Data analyses was performed with Thermo Scientific™ BioPharma Finder™ 5.2 software.

Results: A short chromatographic separation technique was used, which resulted in an elution order that corresponded to the length of the digestion fragments. The Orbitrap Astral mass spectrometer, employing a DDA method with stepped collision energy (CE), was utilized to identify the sequences of the digestion fragments, which ranged from 3 to 60 bases in length. MS/MS matching and annotation techniques were employed to identify the digestion fragments within the digested mRNA sample. Overall, an mRNA sequence mapping coverage of 92% was achieved for the digested sample.

Introduction

Advances in biotechnology and molecular medicine have facilitated the assembly of almost any functional protein or peptide in the human body by introducing mRNA as a therapeutic agent. mRNA-based drugs are geared toward rare diseases, such as genetic disorders, cancers or infectious disease. A well-known example is the response to the Coronavirus pandemic with mRNA-based vaccines. For this reason, there is currently significant demand for the development of new and improved analytical methods for the characterization of mRNA therapeutics. The aim of this work was to develop a workflow covering sample preparation, LC-MS analysis, and data processing to characterize mRNA therapeutics.

Materials and methods

Sample Preparation

A standard mixture of 5 oligonucleotides ranging from 10 to 55 nucleotides bases (**sample A**) was used to perform method optimization.

An mRNA sample consisting of 3500 nucleotide bases (**sample B**) was obtained and sample digestion was performed using immobilized RNase T1 on magnetic beads. mRNA samples were diluted to a concentration of 0.5mg/mL in a digestion buffer and 2.5 µL of the immobilized T1 beads were added. The sample was incubated at 37° C with continuous agitation and evaluated across an incubation time range of 5 to 20 minutes. The reaction was stopped at the designated time point using a magnet to completely remove the magnetic bead resin, followed by centrifugation. An equal volume of 1% formic acid was added to the remaining solution.

LC method

To analyze the resulting sample solution, 0.5 fmol of sample A or a volume of 30 µL of sample B were injected onto a Thermo Scientific DNAPac RP column. The chromatographic separation was carried out using a Thermo Scientific™ Vanquish™ UHPLC system. Mobile phase A was prepared using water containing 0.2 % TEA (triethylamine) and 1 % HFIP (hexafluoroisopropanol). Mobile phase B was prepared using methanol containing 0.2 % TEA and 1 % HFIP. To achieve the chromatographic separation of the mRNA digestion fragments, a 15-minute gradient (for sample A) and a 30-minute gradient (for sample B) were employed. The gradient ranged from 5 % to 20 % mobile phase B. Throughout the analysis, the DNAPac RP column temperature was maintained at 50° C, and the flow rate was set at 300 µL/min.

MS/MS method

The data collection for this study was conducted using a Thermo Scientific Orbitrap Astral mass spectrometer. Sample analysis was carried out using a data-dependent MS/MS acquisition method, along with stepped CE as shown in Figure 1. The experimental conditions for the MS source and mass spectrometer are provided in Table 1 and Table 2, respectively. These tables contain detailed information regarding the settings and parameters used during the analysis.

Figure 1. Stepped CE in Orbitrap Astral mass spectrometer. Three injections with different collision energies are performed. After the ions of all injections are fragmented, they can be transferred to the Astral analyzer and the next scan can be started in parallel. In this example for a total injection time of 6ms, m/z 1500 and a final normalized collision energies of about 28 V, a repetition rate of about 45 Hz can be achieved.

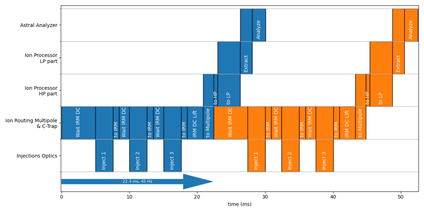


Table 1. MS source conditions.

MS source settings	Value
Spray Voltage (V)	-2500
Capillary temperature (° C)	320
Sheath gas (a.u.)	35
Aux Gas (a.u.)	10
Vaporizer temperature (° C)	300

Table 2. MS scan conditions.

Scan settings	Value
General	
Application mode	Peptide
Polarity	Negative
RF Lens (%)	50
Full Scan	
Scan range (*m/z*)	450-4000
Resolution	120000
AGC target value (%)	300
Max injection time (ms)	50
dd-MS/MS	
Analyzer	Astral
Isolation Window	3
Scan Range	150-2000
AGC target value (%)	100
Max injection time (ms)	10
Normalized collision energy (NCE - V)	18, 23, 28

Data Processing

Data analyses were performed with Thermo Scientific™ BioPharma Finder™ 5.2 software, using the oligonucleotide sequencing workflow.

Results

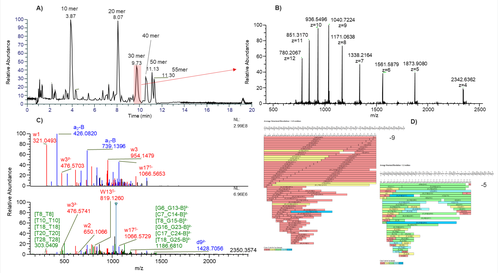
Analysis of oligonucleotides standard mixture

The column and method gradient utilized in this study achieved effective chromatographic separation, successfully resolving all oligonucleotides present in the standard mixture as depicted in Figure 2A. In the context of oligonucleotides LC/MS analysis, fragmentation identification plays a crucial role in confirming the sequence order and providing information regarding potential modifications and their respective sites within the sequence.

Stepped collision energy was chosen to increase the diversity of fragment ions produced. This has proven helpful for peptide analysis[1] and from previous studies on oligonucleotides[2,3]. The data analysis software automatically generated MS/MS fragment identification and annotation, which were color-coded for easy interpretation. Furthermore, the observed fragmentation spectrum was automatically compared to a predicted fragmentation model generated specifically for each identified sequence, as illustrated in Figure 2C.

To evaluate the MS/MS fragmentation identification, the fragment coverage map tool was employed. This tool visually represents all identified fragments and assigns a color-coded intensity to each of them. Figure 2D showcases the sequence coverage map and the average structural resolution (ASR) obtained for two charge states fragmented from the 30 mer oligonucleotide present in the standard mixture.

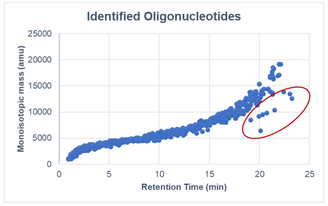
Figure 2. Standard oligonucleotide mixture results. Base-peak chromatogram of oligonucleotide standard mix (A) with full MS spectrum of the 30nt oligonucleotide (B) using stepped collision energy for MS/MS with NCE 13, 16 & 19. Predicted and experimental tandem mass spectra (C). Sequence coverage map for charge state -9 and charge state -5 (D).



Analysis of mRNA sample

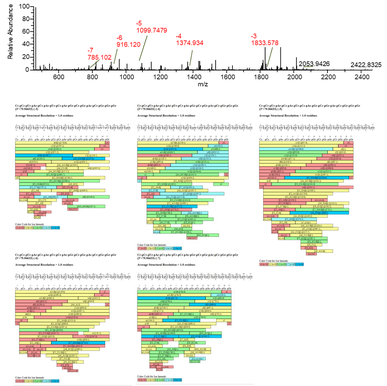
The elution order of the oligonucleotides from the column was observed to be molecular size dependent, with larger digestion fragments exhibiting increased retention time. To verify this elution order trend a plot of molecular weight versus RT was generated (Figure 3). This plot was generated to identify any outliers and provide additional confidence in the identification of digestion fragments and overall data review (outliers are circled in red).

Figure 3. Oligonucleotide molecular weight versus chromatographic elution time for each identified digestion fragment.



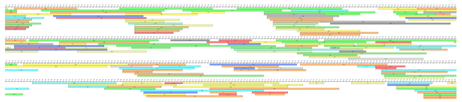
As shown in figure 4, different charge states of the same digestion fragment mRNA show similar results. The software calculates an ASR value, this gives an indication of the number of bonds between each individual nucleotide residue that have been broken and resulted in fragment ions matched to the known sequence. A score of 1.0 indicates that each nucleotide bond in the sequence has been fragmented and identified to the original sequence giving full coverage. The Astral MS2 data show that the charge states from -3 to -7 give full sequence coverage with ideal ASR figures of 1.0, despite the low abundance of some of them. Stepped collision energy in Orbitrap Astral mass spectrometer provided faster and increased diversity of fragment ions which allowed for an enhanced level of confidence for sequence identification and confirmation.

Figure 4. Full scan spectra of a 17 nt and relative oligo sequence coverage maps



The oligonucleotide sequence coverage map was utilized to assess the overall mRNA sequence mapping coverage. Mapping coverage was determined by summing the identified digestion fragments and their respective sequence location assignments within the mRNA sequence (Figure 5). The sequence coverage map provided a percentage value for sequence coverage and was filtered to include only results that met specified criteria. These yielded an overall sequence coverage of 88.2% for unique identifications and 92.4% when including non-unique identifications.

Figure 5. Example selection of the sequence coverage map for the digested mRNA sample. Identified sequence fragments between bases 1201 and 1800 after filter application.



The enhanced speed of the Orbitrap Astral mass spectrometer facilitated the successful sequencing of all the analyzed samples, achieving ASR values close to the desired value of 1.0. In comparison to previous experiments conducted on the same sample using a Thermo Scientific™ Orbitrap™ Exploris™ 240 mass spectrometer, the Orbitrap Astral mass spectrometer enabled the acquisition of 2-3 times more data-dependent MS2 scans. This increased data acquisition capability and allowed for the identification of low abundant impurities associated with some of the oligonucleotides present in the sample. The Orbitrap Astral mass spectrometer offers a DDA analysis speed up to 45 Hz using stepped CE with 3 collision energies, which is 3-4 times faster than observed in previous experiments conducted on oligonucleotides[4].

Conclusions

- A successful sample digestion procedure was developed to achieve reproducible and controlled mRNA digestion utilizing immobilized RNase on magnetic beads.
- The identified digestion fragment sequences in the mRNA digest ranged from 5 to 50 bases in length.
- Stepped collision energy in Orbitrap Astral mass spectrometer provided increase diversity of fragment ions which allowed for an enhanced level of confidence for sequence identification and confirmation.
- Based on the analysis, the overall sequence coverage was determined to be 88% for unique identifications and increased to 92% when including non-unique identifications.
- The Orbitrap Astral mass spectrometer offers a DDA analysis speed up to 45 Hz using stepped CE with 3 steps, which is 3-4 times faster than previously observed[4].

References

1. Diedrich, J. K.; Pinto, A. F. M.; Yates, J. R. Energy Dependence of HCD on Peptide Fragmentation: Stepped Collisional Energy Finds the Sweet Spot. J. Am. Soc. Mass Spectrom. 2013
2. Wei, B.; Wang, J.; Cadang, L.; Goyon, A.; Chen, B.; Yang, F.; Zhang, K. Development of an Ion Pairing Reversed-Phase Liquid Chromatography-Mass Spectrometry Method for Characterization of Clustered Regularly Interspaced Short Palindromic Repeats Guide Ribonucleic Acid. J. Chromatogr. A 2022
3. Vanhinsbergh, C. J.; Criscuolo, A.; Sutton, J. N.; Murphy, K.; Williamson, A. J. K.; Cook, K.; Dickman, M. J. Characterization and Sequence Mapping of Large RNA and mRNA Therapeutics Using Mass Spectrometry. Anal. Chem. 2022
4. Criscuolo, A.; Arrey, T.; Williamson, A. J. K.; Cook, K.; Murphy, K.; Sutton, J.N.; Harder, A.; Shofstahl, J.; Sanders, M. Characterization of mRNA Therapeutics Using a Novel LC-MS Based Workflow. ASMS 2021

Acknowledgements

We would like to acknowledge Tufail Bashir, Cheryl Rhodick and the team in Life technologies for providing the oligonucleotide standards.

Trademarks/licensing