Sequence and impurity analysis of a double-stranded siRNA using a streamlined ion-pair reverse-phase LC-MS/MS workflow

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This technical note highlights a streamlined ion-pair reverse-phase liquid chromatography-tandem mass spectrometry (IP-RP LC-MS/MS) workflow for accurate mass measurement, confident sequence analysis, and rapid impurity assessment of a small interfering RNA (siRNA). This workflow combines excellent chromatographic separation and high-quality MS/MS data of oligonucleotides (oligos) with automated data analysis, leading to rapid intact deconvolution and sequencing of oligos and their impurities (Figure 1).

Therapeutic oligos, such as antisense oligos (ASOs) and siRNA, are promising drug modalities targeting a variety of diseases through various mechanisms of action.[1] IP-RP LC-MS and MS/MS are commonly used to characterize oligos and their impurities. A streamlined workflow—from chromatographic separation to data acquisition and analysis—is essential to enable rapid mass measurement, sequence confirmation, and impurity assessment, accelerating the development and decision-making process of therapeutic oligos.[2-4]

Key features of the streamlined IP-RP LC-MS/MS workflow for oligo analysis

- **High performance**: Oligos are separated using the efficient Biozen Oligo LC column and characterized using the high-resolution ZenoTOF 8600 system, with automated data analysis performed using intuitive Biologics Explorer software
- **Minimal method optimization**: SCIEX OS software allows the user to build a data-dependent CID method using a dynamic CE with custom equation, providing balanced MS/MS fragmentation of oligos in different lengths or charge states
- **Simplicity**: Biologics Explorer software provides easy-to use data analysis workflows for oligo sequence creation, intact deconvolution, mass mapping, and MS/MS sequencing
- **Consistent recovery:** Biozen Oligo LC column combines core-shell particle technology and bioinert hardware, significantly increasing the chromatographic efficiency

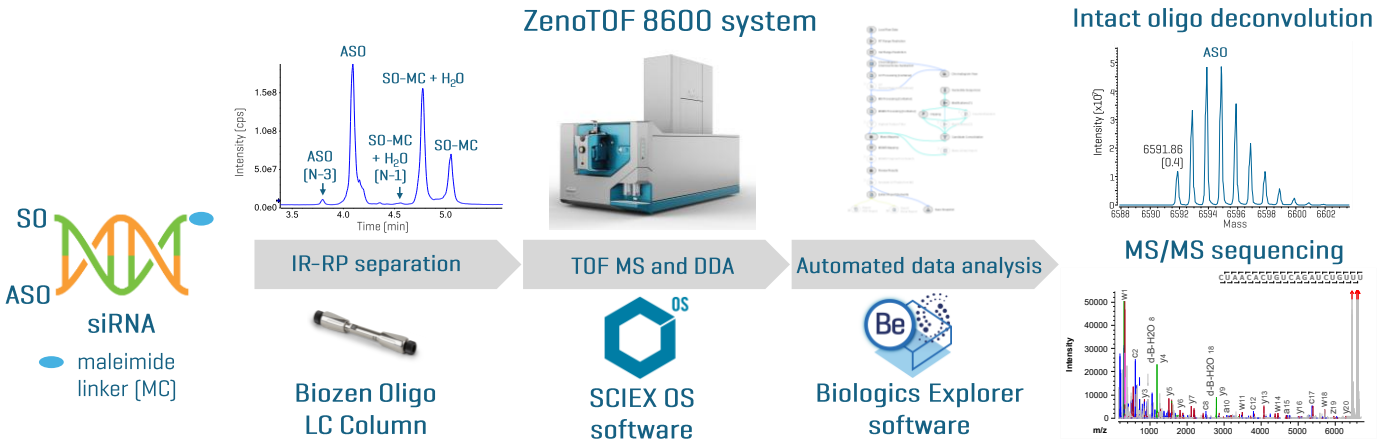

Figure 1. A streamlined IP-RP LC-MS/MS workflow for intact mass and sequence analyses of siRNAs. The maleimide C2 (MC)-linked sense (SO-MC) and ASO of the siRNA are separated based on IP-RP chromatography using a Phenomenex Biozen Oligo LC column, followed by data-dependent acquisition (DDA) in SCIEX OS software using a ZenoTOF 8600 system (shown) or a ZenoTOF 7600+ system (not shown). Oligo deconvolution and MS/MS sequencing are performed using intuitive data analysis workflows within Biologics Explorer software, Version 8.0.3.

Methods

Sample preparation: siRNA with an MC linker conjugated to the 3' end of the SO was purchased from VectorBuilder. A stock solution of 1 mg/mL was prepared for the siRNA. This solution was diluted to 10 ng/µL, from which 5 µL (50 ng) was injected for LC-MS analysis.

IP-RP LC separation: The oligos were separated based on IR-RP chromatography using a Phenomenex Biozen Oligo LC column (2.6 µm, 50 x 2.1 mm) installed on a Nexera XS inert HPLC system (Shimadzu). The separation was performed using the gradient shown in Table 1 with a column temperature of 60°C and a flow rate of 0.4 mL/min. Mobile phases A and B are water and methanol/water (1:1 v/v), respectively, with 50 mM 1,1,3,3,3-hexafluoroisopropanol (Sigma-Aldrich) and 15 mM diisopropylethylamine (Sigma-Aldrich).

Table 1. Gradient for IP RPLC separation.

Time (Min)	Mobile phase A (%)	Mobile Phase B (%)
Initial	90	10
1	90	10
10	50	50
11	5	95
13	5	95
13.1	90	10
15	90	10

Mass spectrometry: MS data were acquired using a DDA method with CID in the negative mode on a ZenoTOF 8600 system (SCIEX) or a ZenoTOF 7600+ system (SCIEX). The key instrument parameters of the ZenoTOF 8600 system are listed in Table 2. The parameters for the ZenoTOF 7600+ system were described previously.[2,3]

Data analysis: MS data were processed using the intuitive oligonucleotide data analysis templates within Biologics Explorer software, Version 8.0.3 (SCIEX). The details on oligo sequence creation, intact deconvolution, and MS/MS mapping were described in previous technical notes.[2,3] Briefly, the sequences of the SO and ASO were defined as the following:

SO

rA-o-rC-o-rA-o-rG-o-rA-o-rU-o-rC-o-rU-o-rG-o-rA-o-rC-o-rA-o-rG-o-rU-o-rG-o-rU-o-rU-o-rA-o-rG-o-rU-o-rU

ASO

rC-o-rU-o-rA-o-rA-o-rC-o-rA-o-rC-o-rU-o-rG-o-rU-o-rC-o-rA-o-rG-o-rA-o-rU-o-rC-o-rU-o-rG-o-rU-o-rU-o-rU

The localized modifications of MC and MC + H_2O were defined to the 3' end of the oligos. The monoisotopic masses of SO-MC and ASO were used for intact deconvolution. All the fragment ion types, such as a, a-B, w, and y, were selected for MS/MS mapping.

Table 2. Source and Zeno CID DDA parameters of the ZenoTOF 8600 system.

Parameter	MS	MS/MS (CID)
Workflow	Intact proteins	
Curtain gas	40	
CAD gas	7	
Ion source gas 1	50 psi	
Ion source gas 2	50 psi	
Temperature	400°C	
Spray voltage	-3,000 V	
Time bins to sum	8	
Declustering potential	-40 V	
Start mass	620 Da	100 Da
Stop mass	3,000 Da	2,000 Da
Accumulation time	0.1 s	0.08 s
Collision energy	-10 V	custom[i]
Zeno pulsing	-	True
Zeno threshold	-	100,000 cps
CE spread	-	0
Max. candidate ions	-	8
Charge states	-	2-12
Q1 resolution	-	Low

[i] A CE equation of CE = 0.0273 × (m/z) + 0.8961 was used for all charge states with the dynamic CE option enabled.[5]

Intact oligo analysis

The Phenomenex Biozen Oligo LC column provides excellent separation of oligos and their impurities. Figure 2A shows the IP-RP separation of the oligo species detected in the MC-linked siRNA. The Biozen Oligo LC column provided baseline separation between the ASO, SO-MC, and their low-abundant impurities. In addition, the hydrated species of the SO-MC (SO-MC + H_2O) was nicely separated from its native form using this column.

Biologics Explorer software offers the intuitive oligo workflows for automated spectrum deconvolution and MS/MS sequencing.[2,3] Intact deconvolution of the siRNA data acquired using the ZenoTOF 8600 system confirmed the identities of ASO, SO-MC, and SO-MC + H_2O (Figures 2B-2D). The measured monoisotopic masses of these species were in excellent agreement with their theoretical values (<2 ppm). Similar results were obtained using the ZenoTOF 7600+ system (data not shown). The impurities annotated in Figure 2A will be described in the following section.

Oligo sequencing

The ZenoTOF 8600 system or the ZenoTOF 7600+ system provides high-quality Zeno CID DDA data of the oligos in the negative mode using fixed CEs with a spread or dynamic CEs with a custom equation.[3] The oligo sequencing workflow within Biologics Explorer software allows a quick comparison of the

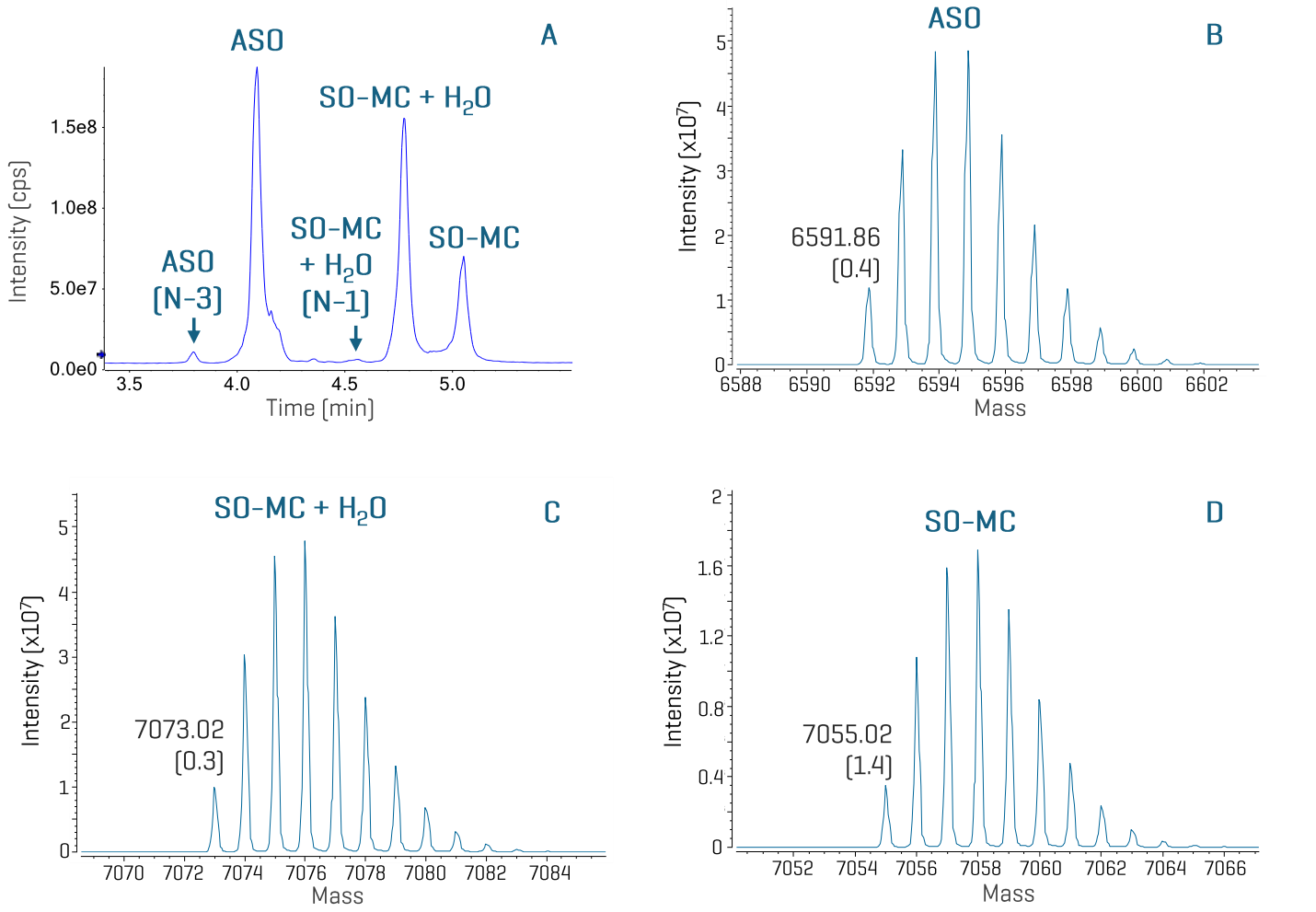

Figure 2. Intact analysis of the MC-linked siRNA. The ASO and SO oligos of the MC-linked siRNA were chromatographically separated using the Biozen Oligo LC column (A). Intact deconvolution using the monoisotopic masses within Biologics Explorer software led to accurate mass determination of these oligos. A high mass accuracy (<2 ppm) was obtained for all the species (B-D). The values in parentheses are the measured mass errors in ppm.

data acquired using different settings, accelerating method optimization for oligo analysis. Figures 3 shows the deisotoped CID spectra and sequence coverage maps of the ASO and SO-MC of the siRNA. Zeno CID DDA with custom dynamic CEs led to excellent fragmentation of these species for their confident sequence confirmation without the need for extensive CE optimization (Figure 3). The MC and MC + H_2O modifications on the 3' end of the SO were confirmed based on the detection of the w- and y-series fragments (Figures 3B and 3C). Similar Zeno CID DDA results were also obtained using the ZenoTOF 7600+ system (data not shown).

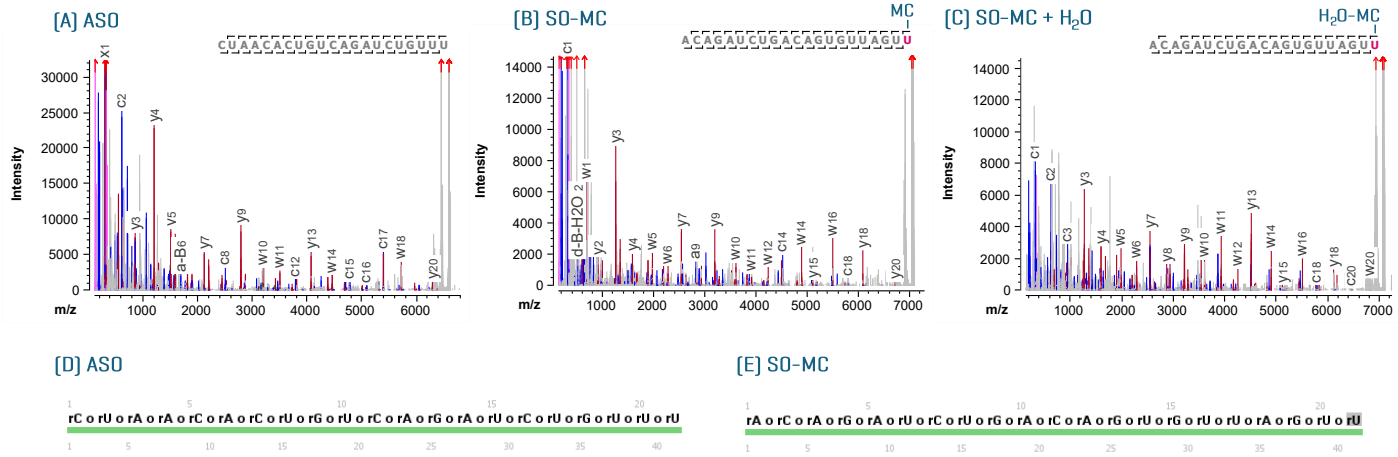

Figure 3. Sequence confirmation of the ASO and SO-MC. CID DDA with the custom dynamic CE setting resulted in excellent fragmentation of the ASO (A), SO-MC (B), and SO-MC + H_2O (C), leading to complete sequence coverage of the ASO and SO (D and E) without the need for CE optimization.

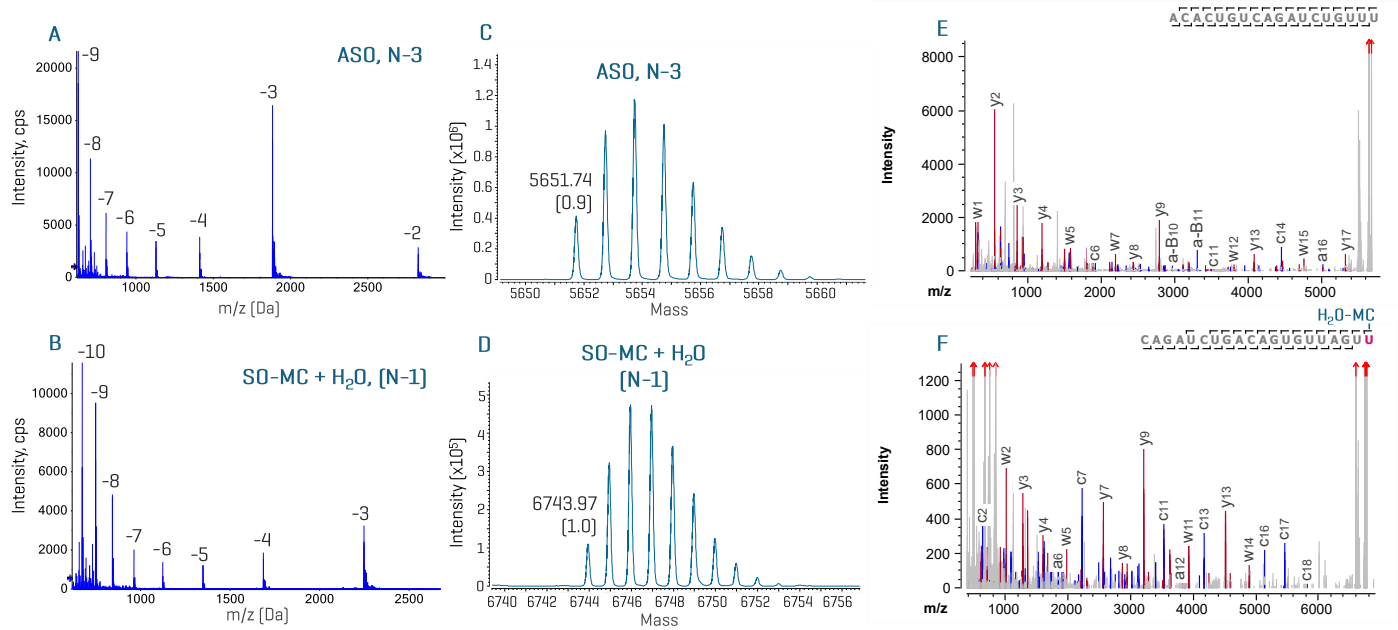

Figure 4. Impurity analysis of the siRNA. Two selected impurities detected in the IP-RP chromatogram (Figure 2A) show a broad distribution of charge states -2 to -10 in their respective mass spectrum (A and B). The deconvolution of these 2 spectra based on monoisotopic masses led to excellent match (<2 ppm) to the theoretical mass of the 5' N-3 species for ASO (C) and the 5' N-1 species for SO-MC + H_2O (D). The sequences of these 2 impurities were further confirmed based on complete sequence coverage obtained using Zeno CID DDA (E and F).

Impurity analysis

The siRNA sample analyzed in this study was purified by HPLC and contained a very low level of impurities. However, the separation power of the Biozen Oligo LC column and the high sensitivity of the ZenoTOF 8600 system enabled the detection and confident identification of low-abundant impurities in the siRNA sample.

Figure 2A shows the presence of low-abundant impurities eluting earlier than the ASO and SO-MC species. Figure 4 shows the raw mass spectra, deconvolution spectra, and annotated MS/MS spectra of 2 selected impurities highlighted in Figure 2. These 2 species were present at ~2.3% and ~0.3% relative to ASO and SO-MC + H_2O, respectively. Intact spectrum deconvolution led to measured masses of 5651.74 Da and 6743.91 Da, in excellent agreement (<2 ppm) with theoretical masses of the 5' N-3 species of AOS and 5' N-1 species of SO-MC + H_2O, respectively (Figures 3A-3D). The identities of these 2 impurities were further confirmed based on a complete sequence coverage obtained from their high-quality Zeno CID DDA data (Figures 3E and 3F).

In summary, this technical note highlights a streamlined IP-RP LC-MS/MS workflow for accurate mass measurement, MS/MS sequencing, and impurity analysis of a synthetic siRNA. The oligo deconvolution and sequencing workflows offered by Biologics Explorer software allow rapid mass measurement, sequence confirmation, impurity analysis, and method optimization, accelerating the development and quality control of oligo therapeutics.

Conclusions

- A streamlined IP-RP LC-MS/MS workflow was developed for intact mass analysis, sequence confirmation, and impurity assessment of synthetic siRNAs using the ZenoTOF 8600 system intuitive Biologics Explorer software. This workflow can be easily replicated on a ZenoTOF 7600+ system.
- Accurate mass measurement (<2 ppm) and high-quality Zeno CID DDA data led to confident identification and sequence confirmation of the ASO and SO species in the siRNA sample.
- The N-3 species at ~2.3% of ASO and the N-1 species at ~0.3% of SO-MC + H_2O were confidently identified based on accurate mass measurement (<2 ppm) and MS/MS mapping.
- Biozen Oligo LC column provided excellent chromatographic separation of oligos and their impurities to facilitate quality assessment.

References

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