POSTER NOTE

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High Resolution LC/MS analysis of Therapeutic Oligonucleotides on a New Porous Polymer-Based Reversed Phase Column

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Overview

Purpose: Demonstrate fast analysis of oligonucleotides (ONs), impurities and structurally modified ONs using ion-pair reversed phase chromatography and ESI-MS.

Methods: Reverse phase separation of ONs were acheived using Thermo Scientific™ DNAPac™ RP column coupled with Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer. TEA/HFIP mobile phases at two different pH values were used for separation of ONs.

Results: ON product, n-1 failure sequence, phosphorothioate, 2'-O-methyl modified siRNA strands and CpG methylated ON were successfully separated and identified by LC/MS using short 3 or 5 minute gradients.

Introduction

Synthetic ONs with different functionalities including antisense ONs, small interfering RNAs (siRNAs), aptamers and immunostimulatory RNAs (isRNAs) are candidate therapeutic agents due to their specificity, and well-established synthesis and modification technologies. Still characterization is required to satisfy regulatory agencies that efficacy and safety of these therapeutic ONs are established. Such analyses include characterization of modifications to the base, sugar and backbone linkages, as these are commonly employed to decrease *in vivo* degradation and increase therapeutic efficacy. High performance LC and LC/MS are the preferred tools for these analyses, and are often used for more common ON purity assessments. Ion-pair reversed phase LC, with volatile mobile phase components, can be directly coupled to MS. Here we introduce a new polymeric reversed phase column and ion-pair methods for LC/MS ON analysis.

Methods

Samples

21mer DNA: GATTGTAGGTTCTCTAACGCT

21mer siRNA sense strand 1: AGCUGACCCUGAAG$_S$UUCAUdCdT

21mer siRNA sense strand 2: A-MeOG-C-MeOU-G-MeOA-s-C-MeOC-C-MeOU-G-MeOA-A-MeOG-s-U-MeOU-C-MeOA-U-dCdT

15mer DNA: CGGCATCCTTATTGG

CpG methylated 15mer DNA: iMe-dC/GGCATCCTTATTGG

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Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:

- SR-3000 Solvent Rack (P/N 5035.9200)
- LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036)
- WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- VWD-3400RS Rapid Separation Variable Wavelength Detector (VWD) equipped with micro flow cell (P/N 5074.0010)
- Chromatography was controlled by Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System .

Mass Spectrometry

The Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer was used for this study. All data were acquired in negative ion mode.

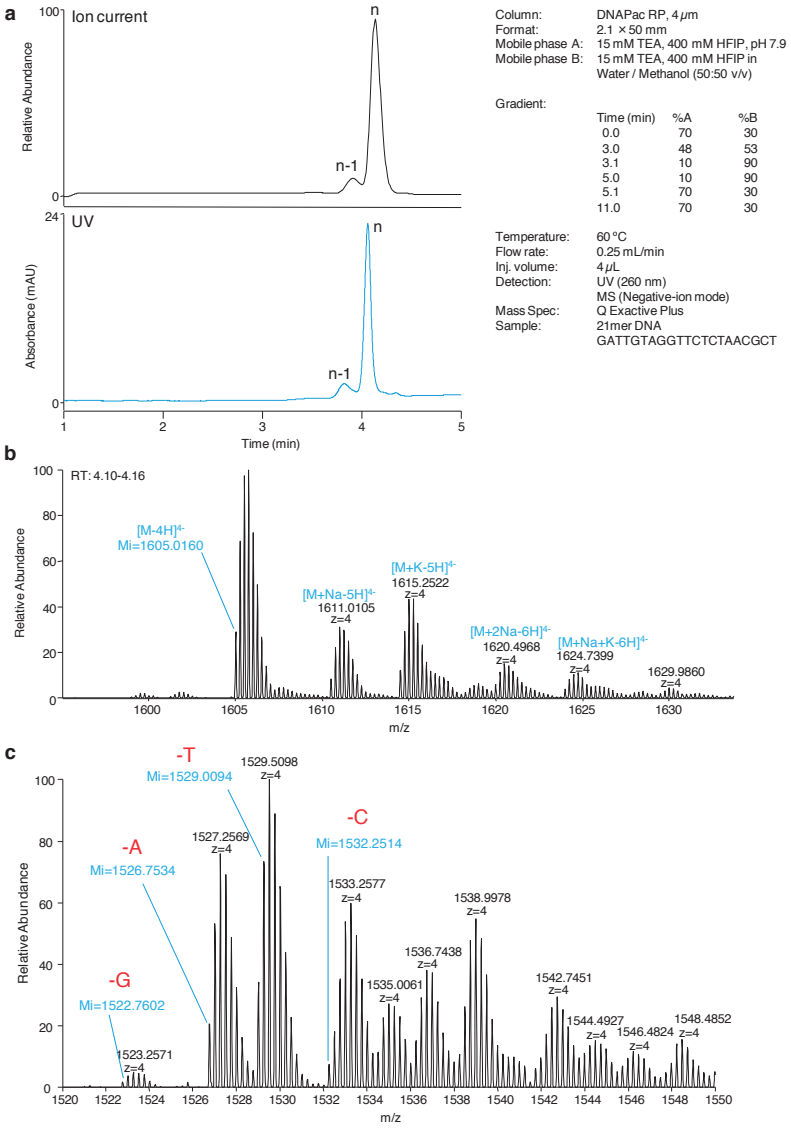
Results

Analysis of failure sequences

Synthetic ONs molecules are used as PCR primers, aptamers, as library adaptors for genomic studies and as therapeutic agents.[1,2] High purity ONs in these applications are required. Therefore separation and identification of failure sequences and other impurities is critical for the production of ON drugs.

In Figure 1a, a 21mer ON was analyzed using mass spec compatible mobile phases (TEA, HFIP). A small peak in front of the target peak was observed. The MS data confirmed the desired target product. Monoisotopic m/z value at charge state -4 for the 21 mer DNA was 1605.016 with mass accuracy of 1.87 ppm (Figure 1b). The high resolution mass spectrometer revealed loss of each of the four bases in the n-1 peak. The masses of failure sequences with missing Guanine or Adenine or Cytosine or Thymine were detected (Figure 1c).

FIGURE 1. LC/MS analysis of failure sequences. a) UV and ion current traces. b) Mass spectrum of 21mer at -4 charge state. c) Mass spectrum of n-1 failure sequence at -4 charge state.

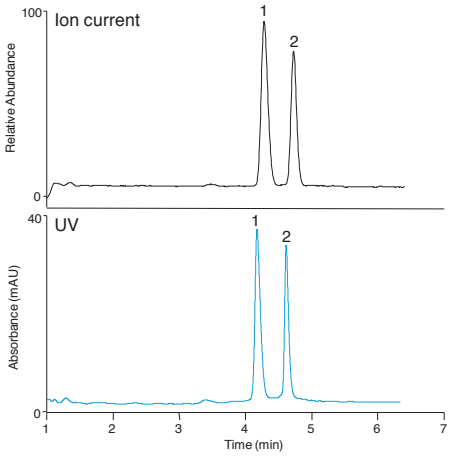


Analysis of phosphorothioate and 2'-O-methyl modified siRNAs

Synthetic siRNAs are important tools for gene function studies and as potential therapeutic agents.[2] Nucleic acids are often modified to increase *in vivo* stability. A common modification in DNA and RNA is incorporation of phosphorothioate (PS) linkages. Another common, but RNA-specific modification is 2'-O-methylation on ribose. The PS linkage introduces a chiral center at phosphorus in addition to the chiral centers in D-ribose of the nucleic acid. Therefore PS modified linkages produce diastereoisomer pairs at each PS linkage.

Figure 2 shows the separation of a sense strand that has one phosphorothioate linkage incorporated at base 14 in the sequence. The two possible diastereoisomers were baseline separated on the DNAPac RP column using high pH mobile phases. At -4 charge state, m/z value of first and the second peaks were 1655.964 and 1655.971 respectively, indicating these molecules to be diastereoisomers rather than failure sequences other impurities.

FIGURE 2. LC/MS analysis of phosphorothioate modified siRNA



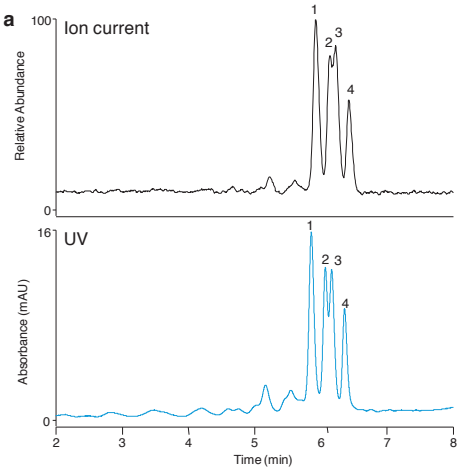
Column: DNAPac RP, 4 μm
Format: 2.1 × 50 mm
Mobile phase A: 35 mM TEA, 40 mM HFIP, pH 9.9
Mobile phase B: 35 mM TEA, 40 mM HFIP in Water / Methanol (75:25 v/v)

Gradient:

Time (min)	%A	%B
0.0	93	7
5.0	52	48
5.1	10	90
7.0	10	90
7.1	93	7
13.0	93	7

Temperature: 30 °C
Flow rate: 0.25 mL/min
Inj. volume: 3 μL
Detection: UV (260 nm)
MS (Negative-ion mode)
Mass Spec: Q Exactive Plus
Sample: 21mer siRNA
AGCUGACCCUGAAG$_s$UUCAUdCdT

FIGURE 3. LC/MS analysis of phosphorothioate and 2'-O-methyl modified siRNA. a) UV and ion current traces. b) Mass spectra of peaks at -4 charge state.

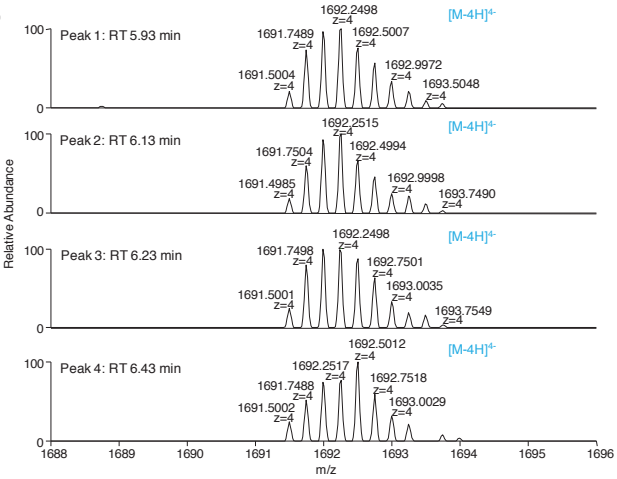


Column: DNAPac RP, 4 μm
Format: 2.1 × 50 mm
Mobile phase A: 35 mM TEA, 40 mM HFIP, pH 9.9
Mobile phase B: 35 mM TEA, 40 mM HFIP in Water / Methanol (75:25 v/v)

Gradient:

Time (min)	%A	%B
0.0	67	33
5.0	42	58
5.1	10	90
7.0	10	90
7.1	67	33
13.0	67	33

Temperature: 30 °C
Flow rate: 0.25 mL/min
Inj. volume: 3 μL
Detection: UV (260 nm)
MS (Negative-ion mode)
Mass Spec: Q Exactive Plus
Sample: 21mer siRNA
A-MeOG-C-MeOU-G-MeOA-s-C-MeOC-C-MeOU-G-MeOA-A-MeOG-s-U-MeOU-C-MeOA-U-dCdT

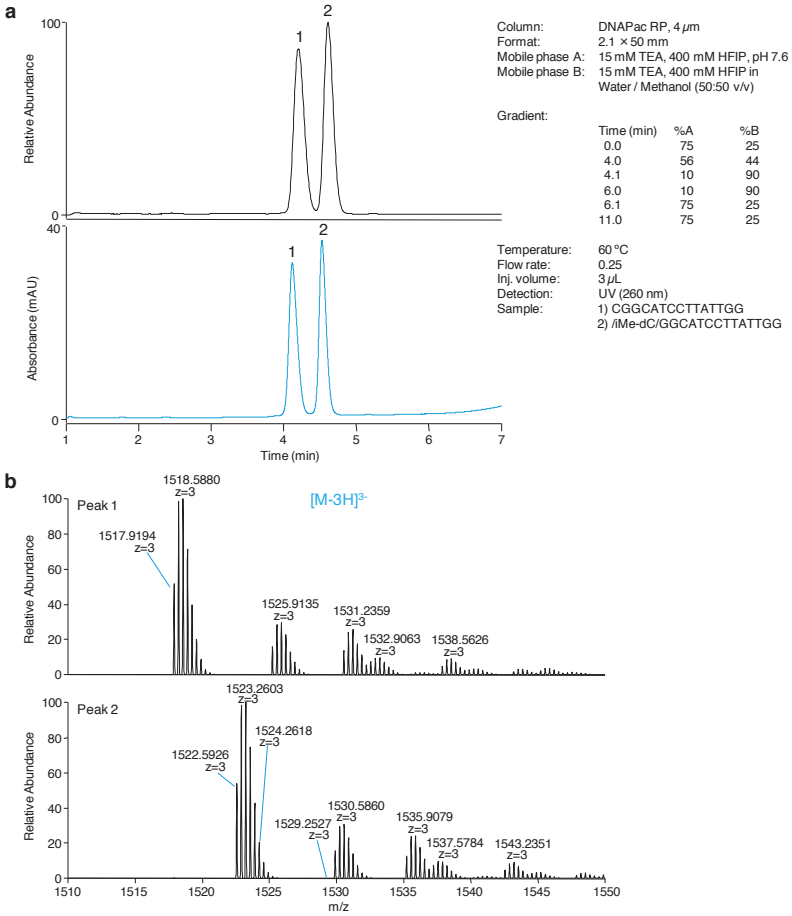


In Figure 3, sense strand of the siRNA was 2'-O-methylated on alternate bases and contains phosphorothioate linkages at the 6th and 14th bases. The UV trace and the ion current traces show the separation of all four possible phosphorothioate diastereoisomers. The high resolution MS data reveal identical masses for all four peaks confirming these molecules to be isomers.

Analysis of phosphorothioate and 2'-O-methyl modified siRNAs

Methylation of CpG sequences in the promoter regions suppresses the expression of the gene and aberrant methylation has been implicated in the development and progression of cancer.[3] Therefore detection of CpG methylation is important for epigenetics studies and cancer research. In Figure 4, an unmodified ON and the CpG methylated ON are well resolved on the DNAPac RP column. Figure 4b shows the -3 charge state of unmodified CpG ON at m/z 1517.919 and the -3 charge state of methylated CpG ON at m/z 1522.593.The mass difference between the methylated and unmodified peaks corresponds to one methyl group.

FIGURE 4. LC/MS analysis of CpG methylation. a) UV and ion current traces. b) Mass spectra of peaks at -3 charge state.



Conclusions

- ON product and n-1 failure sequence, were separated on the DNAPac RP column. High resolution orbitrap mass spectrometer revealed loss of each of the four bases.
- RNAi ONs harboring diastereomers of phosphorothioate with our without 2'-O-methyl modifications were separated using high pH mobile phases.
- CpG methylation was successfully identified using DNAPac RP and high resolution mass spectrometer.

References

1. Dias, N. et al. *Molecular Cancer Therapeutics* (2002) 1, 347-355.
2. Resnier, P. et al. *Biomaterials* (2013) 34, 6429-6443.
3. Jones, P.A. et al. *Nature Reviews Genetics* (2002) 3, 415-428.

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