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

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ABSTRACT

Therapeutic models for synthetic oligonucleotides (ONs) include gene silencing (siRNA), cellular immunity (immunostimulatory [is]) RNA, inhibitory binding (aptamers). These applications require thorough and effective characterization and quality control of synthetic oligonucleotides to satisfy regulatory agencies. High performance LC and LC/MS are the preferred tools for these analyses, and are often used for more common oligonucleotide purity assessments. Ion-pair reversed phase LC is the preferred method for coupling to MS for these analyses. We introduce a new polymeric reversed phase column and ion-pair methods for LC and LC/MS ON analysis.
The new column is based on a highly stable hydrophobic, polymer resin capable of operation at temperatures up to 100 ºC and pH up to 14. Coupling the column to a Q Exactive Plus Orbitrap mass spectrometer successfully identified overlapping failure sequences, ONs with characteristic methylation (CpG and 2'-O-methylated), phosphorothioate diastereoisomers and 5'-phosphorylated DNAs.

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 liquid chromatography (HPLC) and mass spectrometry (MS) comprise valuable tools for the purity assessment and identification of impurities in oligonucleotide samples. Anion-exchange (AEX) chromatography and ion-pair reversed phase chromatography (IP-RP) are the most commonly used techniques for LC/UV or LC/fluorescence analysis of oligonucleotides. However for LC/MS analysis, AEX chromatography is less popular due to the high salt concentration used in their mobile phases which requires a desalting step before MS analysis. 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.

MATERIALS AND 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

21mer siRNA sense strand 3: AGCUGACCCUGAAGUUCAU$_S$d$_S$dT

15mer DNA: CGGCATCCTTATTGG

CpG methylated 15mer DNA: iMe-dC/GGCATCCTTATTGG

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.

Mass range	Spray voltage	Sheath gas	Auxiliary gas	Capillary temp.	S-lens level
m/z 600-4,000	3.5 kV	40 arb. units	15 arb. units	320 ºC	50

In-source CID	Microscans	AGC target	Maximum IT	Resolution	Probe temp.
0 eV	1	3×10^6	200 ms	70,000	300 ºC

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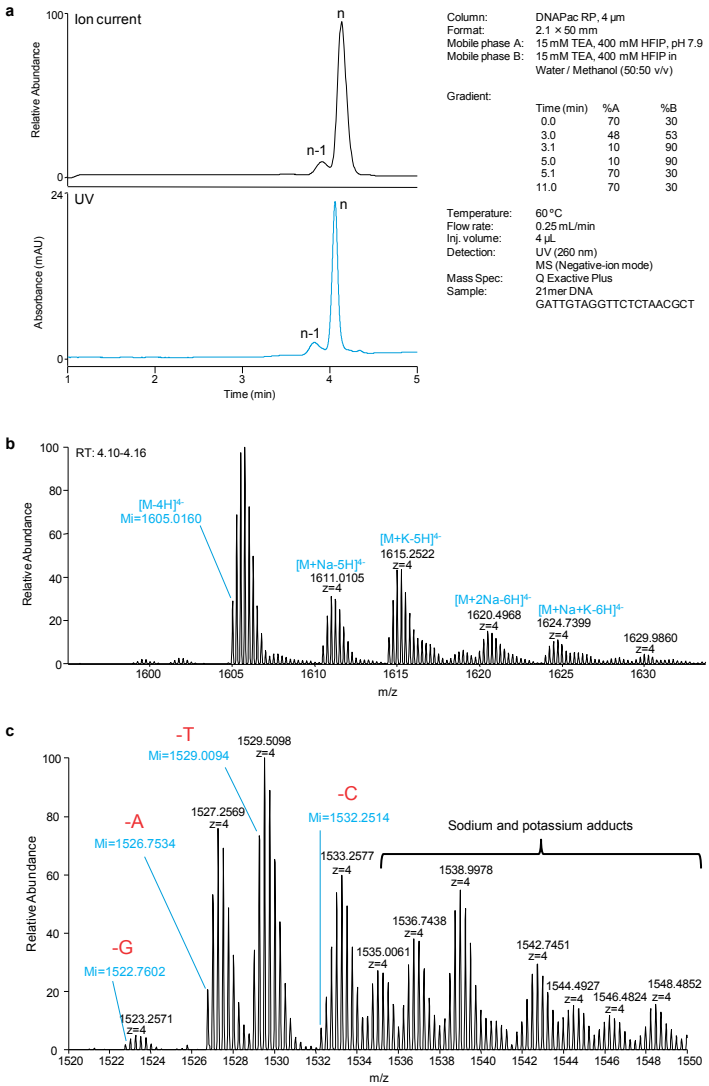
RESULTS

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**).

Figure1. 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.

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.

In **Figure 4**, the sense strand is modified with two PS linkages at the 19th and the 20th bases. In this case, three of the four possible diastereoisomers were chromatographically resolved. In addition to the diastereoisomer peaks, an impurity which contains a single PS linkage (PO) was detected. The mass difference between peak 4 and all three of the other peaks is 16 Da, corresponding to the mass difference between the oxygen and sulfur.

Figure 2. LC/MS analysis of phosphorothioate modified siRNA

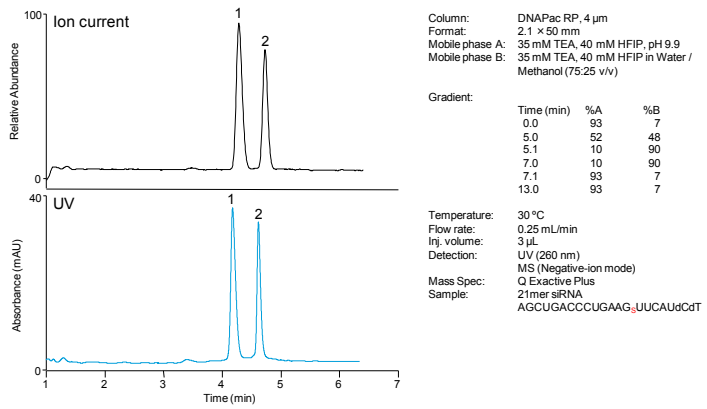


Figure 3. LC/MS analysis of phosphorothioate and 2'-O-methyl modified siRNA. a) UV and ion current traces. b) Mass spectra of peaks at -4 charge state.

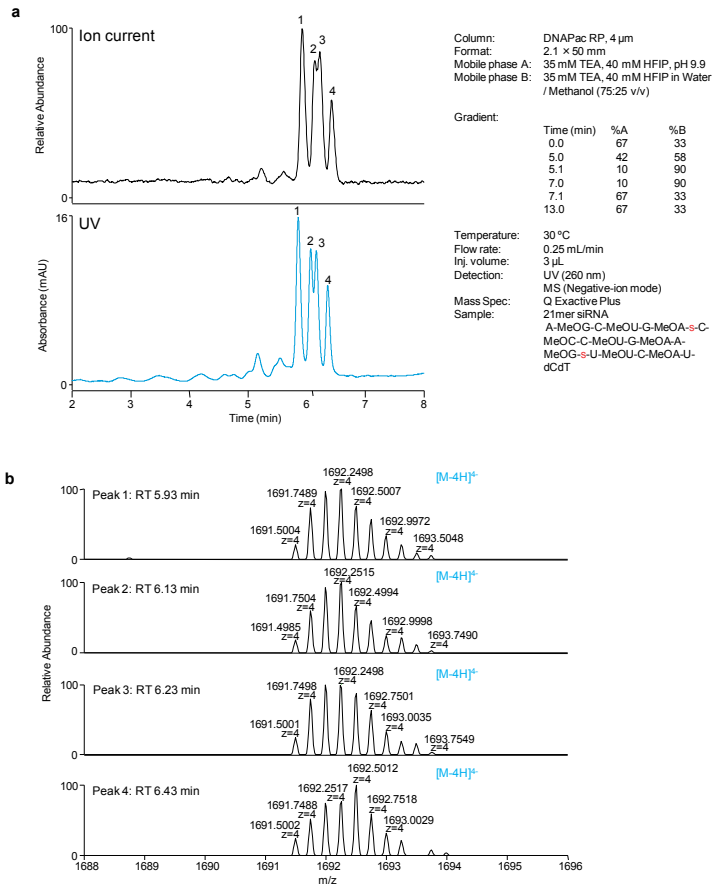
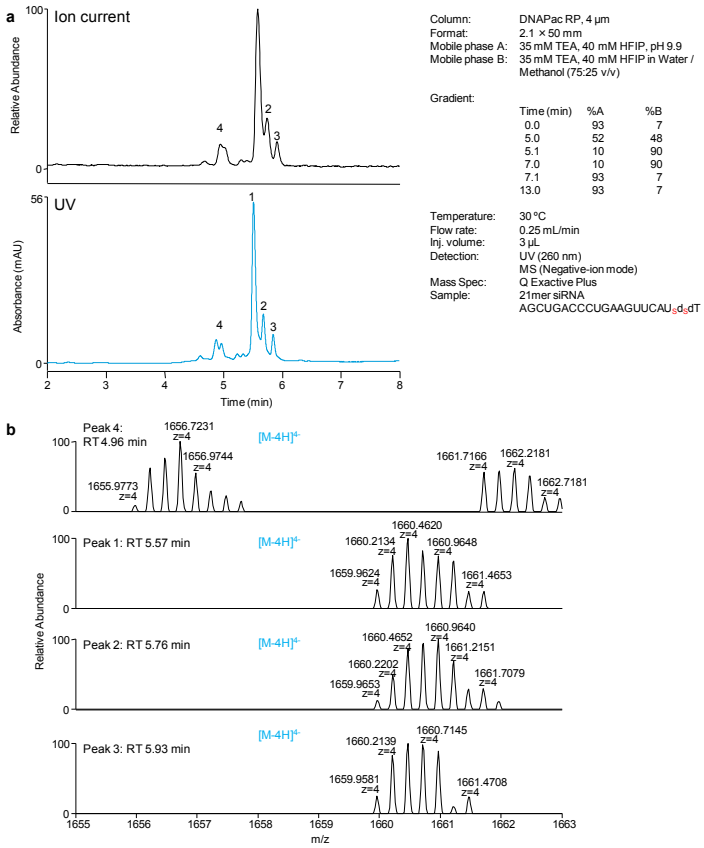


Figure 4. Separation of PO impurity from PS product. a) UV and ion current traces. b) Mass spectra of peaks at -4 charge state.

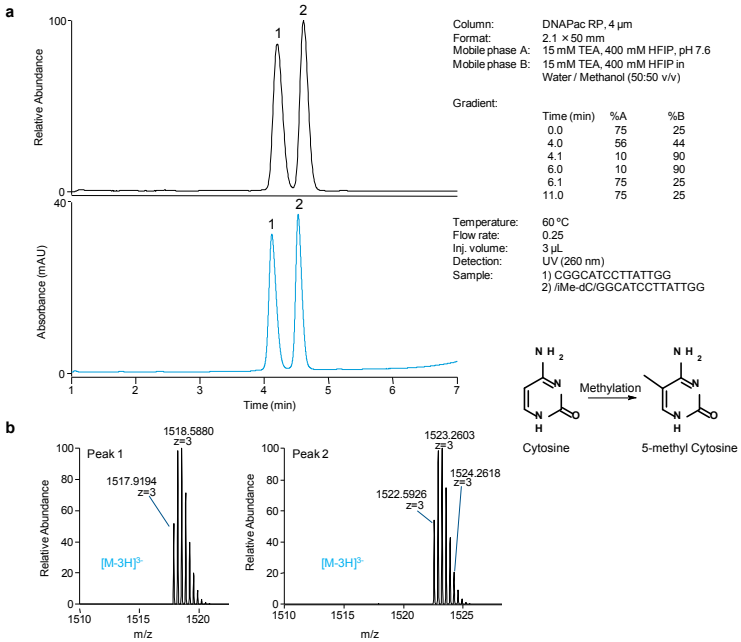


Analysis of CpG methylation

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** 5 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 5. 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.
- siRNA ONs harboring diastereomers of phosphorothioate with our without 2'-O-methyl modifications were separated using high pH mobile phases. In addition, PO impurity was baseline separated from the PS product.
- 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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