UPLC-MS ANALYSIS OF RNAi OLIGONUCLEOTIDES

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INTRODUCTION

Small interfering RNA (RNAi) are oligonucleotides, which selectively disrupt the production of proteins in a process called RNA interference. Interference can be tuned by RNAi sequence selection.

Discovery of RNAi mechanism prompted a need for the analysis of RNAi. To satisfy the need for a robust, fast, and sensitive analysis of 20-25 nucleotides long RNAi, we developed a UPLC/MS method following by MS/MS fragmentation. This approach is suitable for oligonucleotide purity control and monitoring the chemical synthesis efficiency.

METHODS

Instrument

Data were acquired on Waters® Q-Tof Premier and SYNAPT™ HDMS™ mass spectrometers.
Waters® ACQUITY UPLC®, 50 µL mixer (PN 700002631)
ACQUITY UPLC® Photodiode Array detector, 20 points/s sampling rate
MassLynx™ v. 4.1 software for control and data acquisition

RNAi Samples

21 nt (nucleotides): 5' -UUC UGU AAU CUC UUG UCU ATT -3';
20 nt: 5' -UC UGU AAU CUC UUG UCU ATT -3' (Integrated DNA Technologies, Coralville, IA).
All samples were reconstituted in 0.1 M triethylamine acetate (TEAA) to 40 pmole/µL

Column

Oligonucleotide Separation Technology
Waters® ACQUITY UPLC® OST C_{18} 1.7 µm, 135 Å, 2.1x50 mm (PN 186003949)

CHROMATOGRAPHY METHOD

Column temperature: 60°C
Sample injected: 2.5 µL
Sample temperature: 15°C
Flow Rate: 0.2 mL/min
Weak wash: water
Strong wash: water
Mobile Phase:
A: aqueous solution of 15 mM trietylamine (TEA, Fluka 23,962-3) and 400 mM hexafluoroisopropanol (HFIP, Fluka 32,522-8), pH 7.9, measured by weight
B: 50% solvent A, 50% methanol, v/v

Gradient

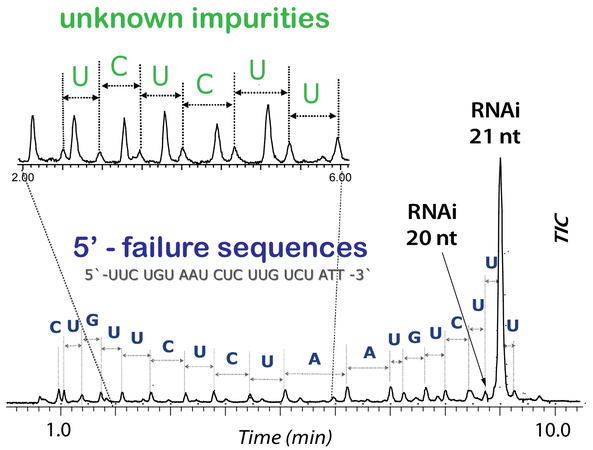
Time	A%	B%
0.00	80.0	20.0
10.00	60.0	40.0
10.10	80.0	20.0
15.00	80.0	20.0

ASSIGNMENT OF FAILURE SEQUENCES

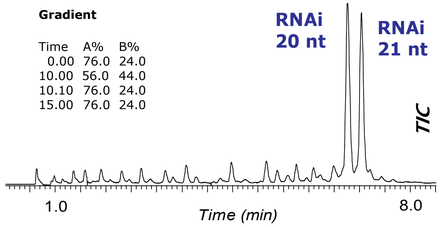
MASS SPECTROMETRY METHOD

Capillary: 2500 V
Desolvation temperature: 200° C
Desolvation Gas Flow: 600 L/hr
Sample cone: 35 V
Source temperature: 120° C
Ion energy: 1 V
Extraction cone: 3 V
Cone Gas Flow: 50 L/hr
TIC detection scan time: 1 sec at 10 sec interval
Ion guide: 2.5 V

MS parameters were chosen in order to achieve maximum declustering of TEA clusters and adducts without compromising ion intensity.

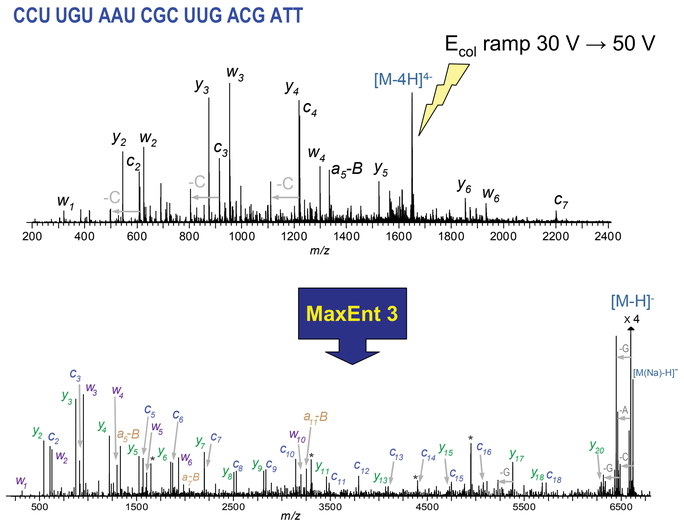


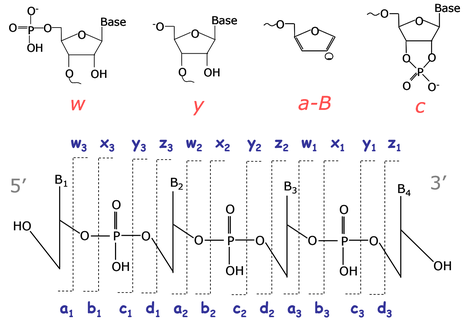
Full length synthetic RNAi was successfully resolved from its failure sequences (5'-truncated oligomers), generated during oligo synthesis. Impurities of another oligomer were recognized. Mobile phases are well compatible with MS-ionization.



21 nt sample was spiked with 20 nt homolog (1:1) to confirm the assignment of the 20 nt peak as a failed sequence.

MS/MS SEQUENCING OF 21-MER RNA





Adopted from McLuckey S.A. *et al.*, *J. Am. Soc. Mass Spectrom.* **1992**, 3, 60-70

ASSIGNMENT OF ION FRAGMENTS

"Lock-Mass" calibration on SYNAPT MS

Calibrant: 10 mg/mL CsI in 50% isopropanol, 50%water
Reference spray flow rate: 5 µL/min
Scan Time: 1 sec, Frequency: 30 sec

ion fragment	theoretical m/z	observed m/z	ppm
w_{20}^-	321.049	321.054	-15.6
$[a_2\text{-}B]^-$	418.066	418.065	2.4
y_{19}^-	545.129	545.127	3.7
c_2^-	609.075	609.074	1.6
w_{19}^-	625.095	625.094	1.6
$[c_3\text{-}Cyt]^-$	804.057	804.057	0.0
y_{18}^-	874.181	874.183	-2.3
c_3^-	915.100	915.102	-2.2
y_{15}^{2-}	926.157	926.144	14.0
c_6^{2-}	935.595	935.585	10.7
w_{15}^{2-}	966.127	966.141	-14.5
y_{14}^{2-}	1098.681	1098.670	10.0
c_7^{2-}	1100.122	1100.114	7.3
$[c_4\text{-}Cyt]^-$	1110.083	1110.085	-1.8
$[a_8\text{-}B]^{2-}$	1157.137	1157.123	12.1
y_{17}^-	1219.229	1219.231	-1.6
c_4^-	1221.126	1221.143	-13.9
y_{13}^{2-}	1251.694	1251.675	15.2
c_8^{2-}	1264.648	1264.639	7.1
w_{17}^-	1299.195	1299.196	-0.8
$[a_5\text{-}B]^-$	1335.157	1335.157	0.0
y_{12}^{2-}	1404.706	1404.690	11.4
c_9^{2-}	1417.660	1417.647	9.2
w_{12}^{2-}	1444.690	1444.683	4.8

ion fragment	theoretical m/z	observed m/z	ppm
$[c_5\text{-}Cyt]^-$	1455.130	1455.131	-0.7
c_{14}^{3-}	1467.164	1467.150	9.5
y_{16}^-	1524.270	1524.269	0.7
y_{11}^{2-}	1557.227	1557.200	17.3
c_5^-	1566.173	1566.174	-0.6
c_{15}^{3-}	1582.180	1582.147	20.9
w_{11}^{2-}	1597.210	1597.198	7.5
$[M\text{-}Gua]^{4-}$	1612.203	1612.161	26.1
y_5^{3-}	1677.897	1677.874	13.7
c_{16}^{3-}	1691.863	1691.863	0.0
w_5^{3-}	1704.552	1704.578	-15.3
y_{10}^{2-}	1729.751	1729.747	2.3
c_{11}^{2-}	1742.705	1742.709	-2.3
w_{10}^{2-}	1769.734	1769.729	2.8
y_4^{3-}	1792.913	1792.956	-24.0
y_{15}^-	1853.323	1853.330	-3.8
y_9^{2-}	1882.271	1882.255	8.5
c_{12}^{2-}	1895.225	1895.229	-2.1
c_8^{3-}	1908.561	1908.528	17.3
w_{15}^-	1933.289	1933.289	0.0
c_{19}^{3-}	2018.245	2018.193	25.8
y_8^{2-}	2035.284	2035.298	-6.9
c_{13}^{2-}	2048.238	2048.242	-2.0
c_7^-	2201.251	2201.272	-9.5

CONCLUSIONS

- **We have demonstrated the ability of the UPLC/MS system to perform routine analysis and quality control of the small interfering RNA molecules. The speed of ACQUITY UPLC will increase the productivity of any laboratory performing oligonucleotides analysis.**
- **Superior UPLC resolution, together with high mass accuracy of Q-Tof Premier detection, allows oligonucleotide purity determination and deciphering of the whole RNAi oligonucleotide sequence, including extra uridine mononucleotide.**
- **MS/MS fragmentation of 21 nt RNA provided an extensive range of the characteristic *y* and *c* ions, sufficient to interpret a full RNAi sequence. Efficient confirmatory sequencing provides verification of known structures according to FDA requirements for therapeutic oligonucleotides.**

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