SHIMADZU

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Rapid monitoring of the purification process of oligonucleotide impurities using a benchtop MALDI-TOF MS system

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1. Introduction

- **Oligonucleotide therapeutics (OTs)** are one of the emerging modalities with high market growth potential. OTs are mainly produced by solid-phase synthesis using the **phosphoramidite chemistry**, which can introduce a variety of **sequence-related impurities** during the synthesis process.
- It is therefore necessary to confirm that these impurities are removed in the purification process at the manufacturing site.
- **LC/MS** is generally used to analyze product-related impurities; however, there are limitations in this approach considering the time frame required for data acquisition and interpretation.
- An alternative approach is to apply **MALDI-MS** as a rapid and simple analytical tool for monitoring the purification process of oligonucleotide therapeutics.

2. Methods

Analyte

- We analyzed a synthetic antisense agent (“**Model MOE-ASO 18 mer**”) which replicates an OT sequence. A crude product of the oligonucleotide was purified by ion-exchange chromatography.

Table 1 Oligonucleotide analyte used in this study

Name	Chemical formula	Mw	Note
Model MOE-ASO 18 mer	$C_{234}H_{349}N_{61}O_{128}P_{17}S_{17}$	7127.2	ASO, PS (full), 2'-MOE, 18 mer
Sequence:	5'-T*-mC*-A*-mC*-T*-T*-T*-mC*-A*-T*-A*-A*-T*-G*-mC*-T*-G*-G*-3'		

* = 2'-O-(2-methoxyethyl) m = 5-methyl d = 2'-deoxy

Mass Spectrometry

- **MALDI-8030** benchtop MALDI-TOF mass spectrometer (Shimadzu) using THAP as a matrix. Sample preparation was optimized to maximize reproducibility.

Software Assignment

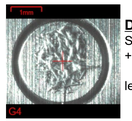
- MALDI-detected impurities of crude/purified samples were assigned by **LabSolutions Insight Biologics version 2.0** (Shimadzu), an oligonucleotide sequence characterization software.

Software for Oligonucleotide Sequence Characterization
LabSolutions Insight™ Biologics

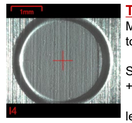
3. Results

Optimization of Sample Preparation for Impurity Profiling

- MALDI-MS is often recognized as lacking in accurate quantitation and reproducibility. This is mainly due to the **inhomogeneity of the crystallized sample/matrix spot** on the MALDI plate.
- The **THAP matrix** proved to be the current best matrix for the impurity profiling. In addition, the use of a new two-layer method combining the traditional dried-droplet and thin-layer approaches, greatly improved quantitation and reproducibility.



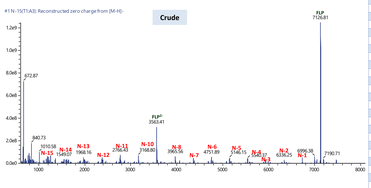
Dried Droplet
Sample solution 0.5 µL
+ Matrix solution 0.5 µL
↓
leave to dry



Two-Layer method
Matrix EtOH solution is deposited to form matrix microcrystals.
↓
Sample solution 0.5 µL
+ Matrix solution 0.5 µL
↓
leave to dry

Crude Sample Analysis

- Fig. 1 shows a MALDI mass spectrum of the crude sample (before purification). Shortmer impurities, together with their thiophospho-adducts were observed.
- The validity of the impurity assignments was also confirmed by LC/MS/MS using a Q-TOF MS “LCMS-9050” and LabSolutions Insight Biologics software (data not shown).



#	Peak	Mass	Height
1	N-15(T1:A3)	1113.52	27026822
2	N-15(T1:A3)[3'Phosphorothiolation]	1209.03	58555173
3	N-14(C15:G18)	1549.07	35583945
4	N-14(T1:C4)[3'Phosphorothiolation]	1603	37265237
5	N-14(C15:G18)[5'Phosphorothiolation]	1645.14	32316187
6	N-13(T1:T5)	1901.39	20199706
7	N-13(G14:G18)	1968.16	53489999
8	N-13(T1:T5)[3'Phosphorothiolation]	1997.29	31706515
9	N-13(G14:G18)[5'Phosphorothiolation]	2064.56	21386280
10	N-12(T1:T6)	2295.43	18558665
11	N-12(T13:G18)	2362.37	35105985
12	N-12(T1:T6)[3'Phosphorothiolation]	2391.36	34994723
13	N-12(T13:G18)[5'Phosphorothiolation]	2458.65	21428134
14	N-11(T1:T7)	2689.63	18467401
15	N-11(A12:G18)	2766.43	73245058
16	N-11(T1:T7)[3'Phosphorothiolation]	2785.38	32237627
17	N-11(A12:G18)[5'Phosphorothiolation]	2862.42	26925407
18	N-10(T1:C8)	3082.58	15605672
19	N-10(A11:G18)	3168.8	75408619
20	N-10(T1:C8)[3'Phosphorothiolation]	3177.9	25343000
21	N-10(A11:G18)[3'Phosphorothiolation]	3264.91	12780368
22	N-9(T1:A9)	3485.78	12111297
23	N-9(T10:G18)	3563.41	320406445
24	N-9(T1:A9)[3'Phosphorothiolation]	3581.74	28901981
25	N-9(T10:G18)[5'Phosphorothiolation]	3658.89	17294344
26	N-8(T1:T10)	3879.61	12118487
27	N-8(A9:G18)	3965.56	61019011
28	N-8(T1:T10)[3'Phosphorothiolation]	3974.42	26968113
29	N-8(A9:G18)[5'Phosphorothiolation]	4061.71	24469967
30	N-7(T1:A11)	4282.51	13534671
31	N-7(C8:G18)	4359.09	49452111
32	N-7(T1:A11)[3'Phosphorothiolation]	4377.84	26414972
33	N-7(C8:G18)[5'Phosphorothiolation]	4454.81	7426694
34	N-6(T1:A12)	4685.32	19187838
35	N-6(T7:G18)	4751.89	54681509
36	N-6(T1:A12)[3'Phosphorothiolation]	4780.73	25315849
37	N-6(T7:G18)[5'Phosphorothiolation]	4848.62	5893937
38	N-5(T1:T13)	5079.32	21283101
39	N-5(T6:G18)	5146.15	48582571
40	N-5(T1:T13)[3'Phosphorothiolation]	5174.67	23006576
41	N-5(T6:G18)[5'Phosphorothiolation]	5242.79	5193844
42	N-4(T1:G14)	5498.19	8520290
43	N-4(T5:G18)	5540.37	45406590
44	N-4(T1:G14)[3'Phosphorothiolation]	5593.59	10464035
45	N-4(T5:G18)[5'Phosphorothiolation]	5636.66	12129733
46	N-3(T1:C15)	5891.49	10976032
47	N-3(C4:G18)	5933.56	31281276
48	N-3(T1:C15)[3'Phosphorothiolation]	5987.03	11077326
49	N-3(C4:G18)[5'Phosphorothiolation]	6029.63	10591710
50	N-2(T1:T16)	6285.44	7835882
51	N-2(A3:G18)	6336.25	31098785
52	N-2(A3:G18)[5'Phosphorothiolation]	6432.75	8984028
53	N-1(C2:G18)	6728.91	53264859
54	N-1(T1:G17)[3'Phosphorothiolation]	6799.95	6192485
55	N-1(C2:G18)[5'Phosphorothiolation]	6825.3	1342954
56	FLP(T1:G18)	7126.81	124833367

Fig. 1 MALDI mass spectrum of the crude oligonucleotide. Due to mass discrimination and the difference in ionization efficiency, MALDI peak height do not directly indicate impurity contents. Peak assignment was carried out by using Labsolutions Insight Biologics version 2.0.

Purified Sample Analysis: Batch purification

- The crude sample was purified with a purity criterion of ≧93% using a single ion exchange column. **Fraction 7** met the criteria*.

*Purity was confirmed by RP-IP analysis.

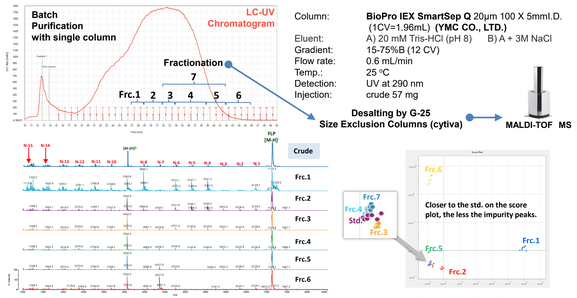


Fig. 3 MALDI mass spectra of the batch purification samples and the result of their principal component analysis (PCA) using eMSTAT Solution™ (Shimadzu). Eight MALDI mass spectra per one fraction were used for the PCA.

Purified Sample Analysis: Continuous Chromatography

- The crude sample was then purified using a continuous chromatography system equipped with two columns (YMC CO., LTD, Japan).
- Using this system, purified samples (cycle 1-10) were obtained.

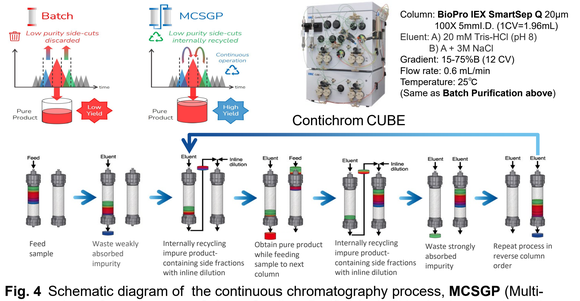


Fig. 4 Schematic diagram of the continuous chromatography process, **MCSGP** (Multi-column Counter-current Solvent Gradient Purification). This simultaneously achieves high purity and yield due to the internal recycling of side-cut.

- Figure 5 summarizes the relative intensities of major impurities (N-1~N-15) before and after MCSGP. The intensities of shortmers were normalized to the intensity of FLP [M-H]⁻. Especially small shortmer impurities could be removed efficiently.
- The impurity profiles of each purification cycle are very similar, indicating that the continuous chromatography purification system works stably. MALDI-MS also showed that the impurity profile of cycle1 was slightly different.

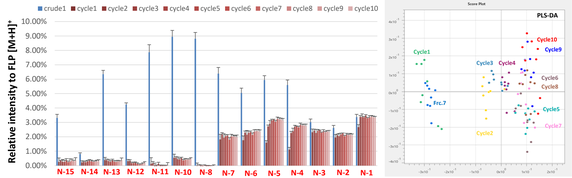


Fig. 5 Relative intensities of major impurities to FLP. Due to mass discrimination and the difference in ionization efficiency, the relative intensities do not directly indicate impurity contents. PCA was performed by eMSTAT Solution.

4. Conclusion

- We investigated a rapid and reproducible profiling method for oligonucleotide therapeutics impurities using MALDI-TOF MS and applied the method to confirm the effectiveness of an oligonucleotide purification process.
- Due to the rapidity and reproducibility of the measurement, we believe it can be easily used to in-process control purpose.

5. Acknowledgements

- This study was supported by AMED under Grant Number JP21ae0121022, JP21ae0121023, JP21ae0121024 (Project leader: Satoshi Obika).
- The Crude oligonucleotide sample used in this study was synthesized by KNC Laboratories Co., Ltd.
- Oligonucleotide purification using continuous chromatography was conducted by YMC CO., LTD.