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Synthetic modified oligonucleotides analysis using a matrix-assisted laser desorption/ionization digital-ion-trap mass spectrometer (MALDI-DIT-MS)

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1. Overview

- In the analysis of oligonucleotide therapeutics, the development of analytical techniques using LC-MS is accelerating, while the need for simpler and faster analytical techniques is also increasing.
- We report here a simple and rapid analytical method of synthetic modified oligonucleotides using MALDI-digital ion trap-MS（MALDI-DIT-MS).

2. Methods

2-1. Analytes (model oligonucleotide samples)

- **Single-stranded DNA**: Mipomersen (GeneDesign Inc.), MW 7177

5' MG-MC-MC-MU-MC-dA-dG-dT-dC-dT-dG-dC-dT-dT-dC-MG-MC-MA-MC-MC 3'

A, C, G, and U represent adenosine, cytidine, guanosine, and uridine deoxyribonucleotides.; M: 2'-O-(2-methoxyethyl) nucleotide d: 2'-deoxynucleotide; Substitution at 5-position of cytosine and uracil base with a methyl group.

- **Single-stranded RNA**: Patisiran, sense strand (GeneDesign Inc.), MW 6764

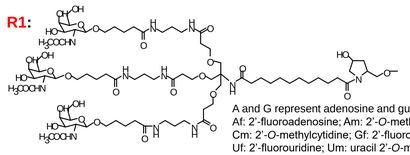
5' G-Um-A-A-Cm-Cm-A-A-G-A-G-Um-A-Um-Um-Cm-Cm-A-Um-dT-dT 3'

- **Double-stranded RNA**: Vutrisiran (MedChemExpress LLC), MW 16345 (sense strand: 8789; antisense strand: 7558)

5' Ums-Gms-Gm-Gm-Am-Um-Uf -Um-Cf - Af - Uf - Gm-Um-Am-Am-Cm-Cm-Am-Am-Gm- Am-R1 3'

3' Cms-Ums-Am - Cm - Cm-Cm-Um -Af- Am-Af -Gm-Um-Am-Cm- Af - Um-Um-Gf -Gm-Um-Um-sCf-sUm 5'

R1:



A and G represent adenosine and guanosine ribonucleotides.
Af: 2'-fluoroadenosine; Am: 2'-*O*-methyladenosine; Cf: 2'-fluorocytidine; Cm: 2'-*O*-methylcytidine; Gf: 2'-fluoroguanosine; Gm: 2'-*O*-methylguanosine; Uf: 2'-fluorouridine; Um: uracil 2'-*O*-methyluridine; dT: thymidine deoxyribonucleotide; R1: triantennary GalNAc, s: Phosphodiester bonds between nucleotides are substituted with phosphorothioate bonds.

2-2. Preparation

1. An aqueous solution of the analyte and a matrix solution: 40 mg/mL 50% ACN/water (v/v) with diammonium hydrogen citrate were prepared.
2. After mixing the analyte solution and the matrix solution at 1:1 (v/v), 1 μL was dropped on the sample plate.

MS (sgl) MS (dbl) MS (seq) MS^n

2-3. MALDI-MS measurement

- Instrument: MALDImini™-1 (Shimadzu Corp.) (Fig. 1)
- Measurement: Raster scan, Positive ion mode

Fig. 1 MALDImini™-1 (MALDI-DIT-MS)

3. Results

3-1. Single-stranded DNA

- **MW analysis**

MS (sgl)

Single-stranded DNA was detected as $[M+H]^+$ with high sensitivity under the analytical conditions in Table 1.

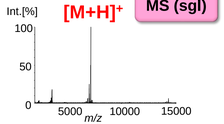


Fig. 2 Mass spectrum of mipomersen (10 pmol/well) using the MW analysis conditions in Table 1.

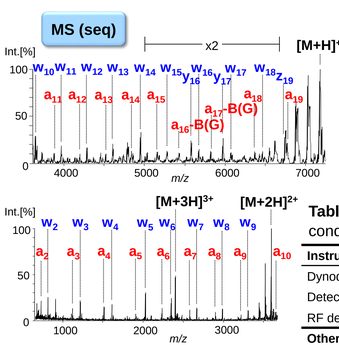
Table 1. MW analysis conditions for single-stranded DNA

Instrumental:		
Dynode voltage (V)	7000	
Detector voltage (V)	1300	
RF delay (μs)	25	
Others:		
Matrix (ex.)	3-HPA/2,4-DHAP	for MW
ACD (concentration)	70 mM	

- **Sequence analysis**

5'- MG MC MC MU MC dA dG dT dC dT dG dC dT dT dC MG MC MA MC MC -3'

MS (seq)



Base sequence information including the modification site was obtained under the analytical conditions in Table 2. In this case, *a/w*-ions were mainly detected.

Table 2. Sequence analysis conditions

Instrumental:		
Dynode voltage (V)	8000	High
Detector voltage (V)	1800	High
RF delay (μs)	17	Low
Others:		
Matrix (ex.)	3-HPA/2,4-DHAP	for seq.
ACD (concentration)	70 mM	

Fig. 3 Mass spectra of mipomersen (10 pmol/well) using the sequence analysis conditions in Table 2.

3-2. Single-stranded RNA

- **MW analysis**

MS (sgl)

Single-stranded RNA was detected as $[M+H]^+$ with high sensitivity under the analytical conditions in Table 3.

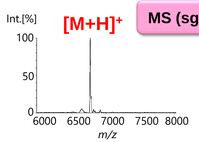


Fig. 5 Mass spectrum of patisiran, sense strand (10 pmol/well) using the MW analysis conditions in Table 3.

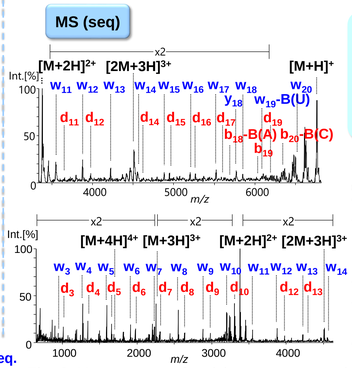
Table 3. MW analysis conditions for single-stranded RNA

Instrumental:		
Dynode voltage (V)	7000	
Detector voltage (V)	1300	
RF delay (μs)	25	
Others:		
Matrix (ex.)	3-HPA/2,4,6-THAP	for MW
ACD (concentration)	70 mM	

- **Sequence analysis**

5'- G Um A A Cm Cm A A G A G Um A Um Um Cm Cm A Um dT dT -3'

MS (seq)



Base sequence information including the modification site was obtained under the analytical conditions in Table 4. In this case, *d/w*-ions were mainly detected.

Table 4. Sequence analysis conditions

Instrumental:		
Dynode voltage (V)	8000	High
Detector voltage (V)	1800	High
RF delay (μs)	17	Low
Others:		
Matrix (ex.)	2,4-DHAP	for seq.
ACD (concentration)	70 mM	

Fig. 6 Mass spectra of patisiran, sense strand (10 pmol/well) using the sequence analysis conditions in Table 4.

3-3. Double-stranded RNA

- **MW analysis**

MS (dbl)

Double-stranded RNA was detected as $[M+H]^+$ under the analytical conditions in Table 5.

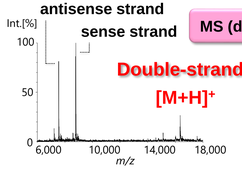


Fig. 7 Mass spectrum of vutrisiran (10 pmol/well) using the MW analysis conditions in Table 5.

Table 5. MW analysis conditions for double-stranded oligonucleotides

Instrumental:		
Dynode voltage (V)	8000	High
Detector voltage (V)	1800	High
RF delay (μs)	80	High
Others:		
Matrix (ex.)	3-HPA	for MW
ACD (concentration)	200 mM	High

- **Sequence analysis**

Base sequence information including the modification site was obtained under the analytical conditions in Table 6.

5' Um Gm Gm Gm Am Um Uf Um Cf Af Uf Gm Um Am Am Cm Cm Am Am Gm Am R1 3'

3' Cm Um Am Cm Cm Cm Um Af Am Af Gm Um Am Cm Af Um Um Gf Gm Um Um Cf Um 5'

MS (seq)

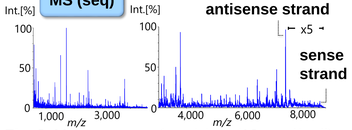


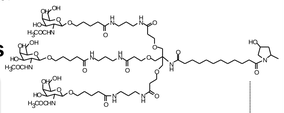
Fig. 8 Mass spectra of vutrisiran (10 pmol/well) using the sequence analysis conditions in Table 6.

Table 6. Sequence analysis conditions

Instrumental:		
Dynode voltage (V)	8000	High
Detector voltage (V)	1800	High
RF delay (μs)	17	Low
Others:		
Matrix (ex.)	2,4-DHAP	for seq.
ACD (concentration)	70 mM	

- **GalNAc modification analysis**

A triplet peak with GalNAc-specific mass difference (203 Da) was observed under the analytical conditions in Table 6. MS^2 and MS^3 of that peak provided structural information for GalNAc terminal modifications.



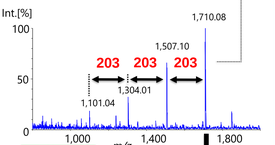


Fig. 9 Enlarged mass spectrum of Fig 8.

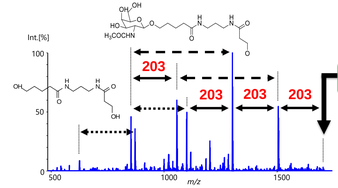
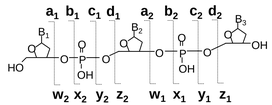


Fig. 10 MS^2 spectrum of the ions at *m/z* 1710 in Fig. 9.



Nucleic acid fragment ion name [1]

[1] McLuckey SA, *J. Am. Soc. Mass. Spectrom.*, 1992, 3, 60-70.

4. Conclusion

We confirmed that molecular weight analysis, base sequence analysis including modification sites, and terminal GalNAc modification analysis for single-stranded DNA/RNA and double-stranded RNA are possible with a single MALDI-DIT-MS instrument.