Method development of a simple and rapid MS analysis of oligonucleotides with novel Acoustic Ejection Mass Spectrometry (AEMS)

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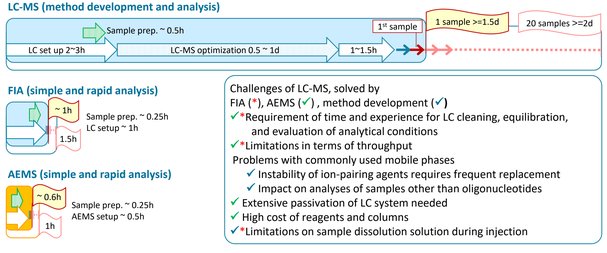
INTRODUCTION-1: Throughput and challenges of LC-MS analysis of oligonucleotides (OGNs)

LC-MS has been established as one of the leading analytical methods for therapeutic oligonucleotides. It can provide high-precision qualitative and quantitative results using LC separation. However, there are some challenges. FIA resolves some of them, but still some remains (Fig.1). This results in wasted time and labor, making it difficult to perform quick and easy analyses or continuous analyses of multiple samples. A new AEMS system is possible to address these challenges.



Challenges of LC-MS, solved by
FIA (*), AEMS (✓) , method development (✓)
✓*Requirement of time and experience for LC cleaning, equilibration, and evaluation of analytical conditions
✓*Limitations in terms of throughput
Problems with commonly used mobile phases
✓ Instability of ion-pairing agents requires frequent replacement
✓ Impact on analyses of samples other than oligonucleotides
✓ Extensive passivation of LC system needed
✓ High cost of reagents and columns
✓*Limitations on sample dissolution solution during injection

Figure 1. Time course, throughput and challenges of LC-MS and FIA of OGNs and expectations for AEMS.

INTRODUCTION-2: AEMS and benefits for OGN analysis

Droplets are ejected from the sample by acoustic energy and delivered to the MS electrode by solvent flow in OPP.

Acoustic Droplet Ejection (ADE):
- Non-contact, high-speed, high accuracy, and high reproducibility sampling in the order of nL

Open-Port Interface (OPI):
- Direct liquid transfer, no sample loss or carry-over
- Significant matrix dilution, allowing the direct ejection of complex samples

Electrospray ionization:
- Spectral equivalence to LC-MS

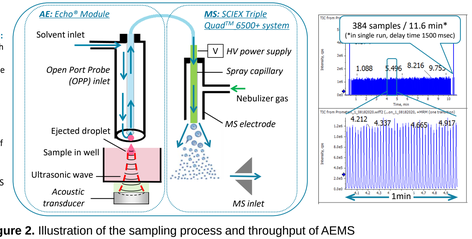


Figure 2. Illustration of the sampling process and throughput of AEMS

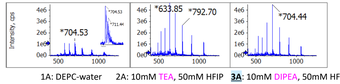
If AEMS is applicable to OGN analysis,
- it will be possible to analyze easily, quickly and at low cost without LC.

If a stable and general solvent can be used as the mobile phase,
- the solvent does not need to be changed frequently, and continuous analysis including samples other than OGN becomes possible.

RESULT-1: Dilution solvents

Ion-pairing agents and organic solvents commonly used in LC-MS were tested as diluents at various concentrations and ratios. Methanol, the default mobile phase for system, was selected, simplifying system setup and avoiding the problems of mobile phases (instability, adduct formation) commonly used in LC-MS for OGNs.

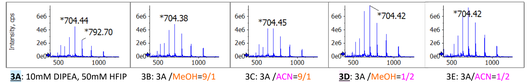
1-1. Ion pairs: TEA+HFIP, DIPEA+HFIP



1A: DEPC-water 2A: 10mM TEA, 50mM HFIP **3A**: 10mM DIPEA, 50mM HFIP

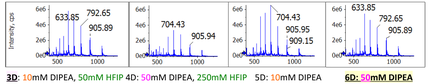
Mobile phase: •Methanol can be used,
• Ion-pairing agents were not required
Diluent: • Amines enhanced sensitivity and reduce adducts ions, • HFIP was not required
• No issues with adding organic solvent and no need to remove organic solvents

1-2. Organic solvents and %ratio



3A: 10mM DIPEA, 50mM HFIP 3B: 3A /MeOH=9/1 3C: 3A /ACN=9/1 **3D**: 3A /MeOH=1/2 3E: 3A/ACN=1/2

1-3. Concentrations of ion pairs (diluted three times with methanol)



3D: 10mM DIPEA, 50mM HFIP 4D: 50mM DIPEA, 250mM HFIP 5D: 10mM DIPEA **6D: 50mM DIPEA**

Figure 3. Comparison of sensitivity and spectra between dilution solutions. The settings selected for subsequent studies are highlighted.

DEPEC: diethylpyrocarbonate, HFIP: hexafluoroisopropanol, TEA: triethyl amine, DIPEA:N-ethyldiisopropylamine

RESULT-2: Concentration and ejection volume

Since alkali metal, commonly found in ONG analysis, reduce sensitivity and increase spectral complexity, there is a desire to limit adduct formation. The effect of concentration and ejection volume on the spectrum and adduct ions were investigated.

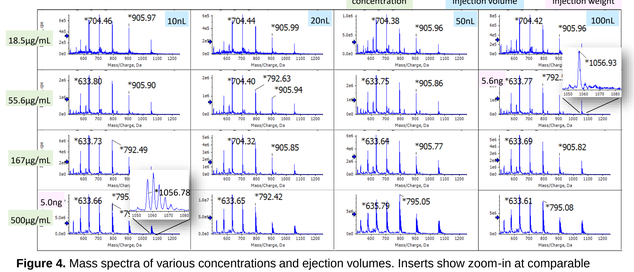


Figure 4. Mass spectra of various concentrations and ejection volumes. Inserts show zoom-in at comparable injection weights.

- There was no significant difference in the charge distribution depending on the concentration
- Dilution in 16.5 mM DIPEA in 67% ACN reduced the adduct ions caused by alkali metals
- A good linearity (R^2>0.99, CV=<10%) was obtained for the peak area values of TIC at each injection volume

RESULT-3: High-throughput analysis of various OGNs

To confirm the same AEMS conditions could be used for various OGNs, all samples were prepared in 16.5 mM DIPEA in 67% ACN at 50-200 μg/mL in different wells on a plate and acquired in a single run.

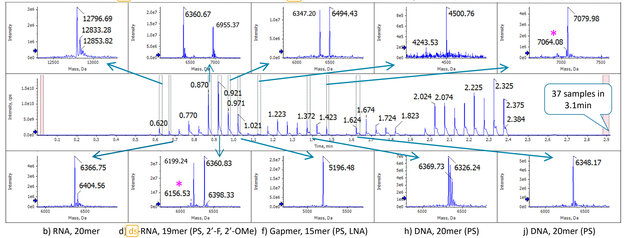


Figure 5. Reconstructed data of various OGNs.

- Molecular weights of all the OGNs could be obtained in less than 4 minutes using the exact same conditions
- The double-stranded (ds) OGNs (sample c, d, e) were detected as two separate chains without prior denaturation
- Impurities (*) were detected in samples d and i. The impurity profile of sample d was similar to the results of LC-HRMS analysis (RESULTS-4)

RESULT-4: Impurity profiling

The impurities detected in sample d were compared with those detected by previous LC-HRMS analysis.

LC-HRMS (Reference Data, 500ng on column, acquired on the TripleTOF 6600 system from SCIEX)

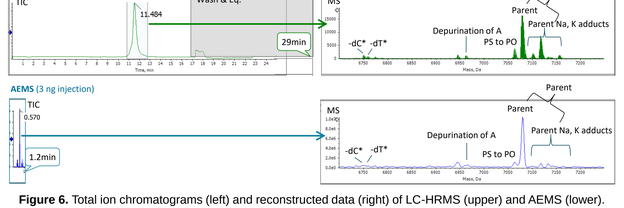


Figure 6. Total ion chromatograms (left) and reconstructed data (right) of LC-HRMS (upper) and AEMS (lower).

- AEMS detected the profiles of major impurities similar to LC-HRMS
- By using AEMS, the acquisition time was reduced to about 1/20 compared to LC-MS

MATERIALS AND METHODS

Samples:
Results-1, 2: 20 mer phosphorothioated (PS) DNA (dA*dT*dC*dG*dA*dC*dT*dC*dT*dC*dG*dA*dG*dC*dG*dT*dT*dC*dT *dC where *: PS), Results-3: Ten synthesized OGNs of various types and modifications (DNA, RNA, Gapmer, siRNA, PS, LNA (l), 2'-Fluoro (f), 2'-Ome (m), Cholesterol TEG (Chol)) and length (15-40mer), Results-4: 22mer PS-DNA (dG*dA*dG*dA*dT*dC*dG*dG*dA*dT*dT*dC*dC*dA*dG *dT*dA*dT*dA*dC*dC*dA)

AEMS system and software:
System: Echo® MS system (coupled with SCIEX Triple Quad 6500+ mass spectrometer, Software: SCIEX OS software with Bio Tool Kit (SCIEX)

		1	2	3	4
		Test	Optimized		
Echo module	Eluent	MeOH			
	Flow rate (μL/min)	400	380		
	Ejection vol. (nL)	5	50	60	
MS	Cur, Gas 1 (psi)	20, 90			
	Gas 2 (psi)	55	65		
	Temperature (°C)	400	500		
	Polarity, Scan type	Negative, Q1			
	Mass range (m/z)	400-1250	500-1250		
	Scan rate (Da/s)	12000			
	IS, DP (V)	-4500, -80			

Table 1. AEMS parameters

CONCLUSIONS

	LC-MS	FIA	AEMS
Separation / purification	✓✓	N/A	N/A
Quantification	✓✓ with separation	✓	✓? need further verification
Identification (MS level)	✓✓✓ accurate mass with HRMS and LC separation	✓✓ accurate mass with HRMS	✓ average mass
Sequencing	✓ with HRMS	✓ with HRMS	N/A
System setup	preparing mobile phase, LC and column equilibration	preparing mobile phase, LC equilibration	✓ auto-purge only
Method development	required	✓ not required	✓ not required
Mobile phase requirements	need ion pairs and fresh preparation	need ion pairs and fresh preparation	✓ methanol
Sample diluent	restricted	✓	✓
Denaturing of OGN	✓ with high column temp.	N/A, need pre-treatment	✓✓? with AE?, need further verification
Sample throughput / hour	✓ <10 samples	✓✓ <50 samples	✓✓✓ <50 samples, individual <1800 samples, batch
Flexibility of system for other assays	N/A, need system clean up	N/A, need system clean up	✓
Running cost	columns, ion pairs for mobile phases	ion pairs for mobile phases	✓

Table 2. Advantages and disadvantages of each analytical method in OGN analysis.

- Using AEMS, a rapid, simple, cost- and time-effective analytical method for OGN was established.
- The method was shown to be applicable to various types of OGNs.
- AEMS detected the profiles of major impurities similar to LC-HRMS in about 1/20th of the time.
- The ability to use ion-pair-free mobile phases enables continuous analysis including samples other than OGNs.
- The system can be applied to urgent and rapid analysis of a large number of samples, such as reaction confirmation during synthesis, development of synthesis and purification methods, and lot management.

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