High-Resolution Ion Mobility Mass Spectrometry (IMMS) for Oligonucleotide Impurity Analysis

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Abstract

Synthetic oligonucleotide therapeutics (ONTs) modulate gene expression or hinder protein function by binding to specific targets, having the potential of regulating proteins considered “undruggable" by small molecules. ONTs are synthesized using solid-phase chemistry which produces product-related impurities that are structurally similar and challenging to analyze by conventional methods such as liquid chromatography-high resolution mass spectrometry (LC-HRMS). Ion mobility (IM) technology enables a separation based on a molecule’s size and shape by measuring its mobility, complementary to chromatographic or mass spectrometric separation. In this study, IM is used to characterize a full-length product (FLP) and some common impurities. Results indicate that some isobaric or isomeric impurities, not separable by LC or MS, can be differentiated by IM at selected charge states.

Introduction

It is critical that the product-related impurities be fully characterized and quantified. LC-HRMS is a powerful technique in oligonucleotide impurity profiling, but characterizing closely eluting isobaric or isomeric compounds that are not separated by either LC or MS is challenging. IM, when paired with LC-MS, provides an additional dimension of separation. The present study uses an IM with a cyclic geometry that measures the drift time (DT) of molecules, and has the ability to perform multiple passes to achieve high-resolution IM separation **(Figure 1)** . This work uses a single pass to establish a baseline for the mobility of structurally-similar oligonucleotides.

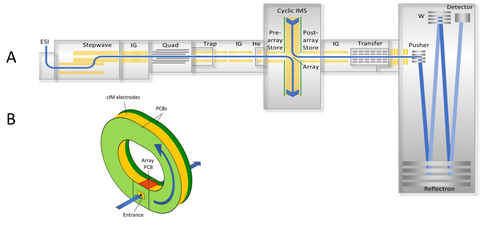

Figure 1. Instrument diagrams of (A) cyclic IM (cIM)-MS used in this study and (B) cIM device. Adapted from [1].

Materials and Methods

A custom-synthesized FLP with the same nucleotide sequence and modifications as nusinersen was used as a model molecule in this study. **Table 1** lists the sequence and molecular weight of the FLP and product-related impurities tested. Experiments were performed using a Waters cIM with an electrospray ionization (ESI) source. Sample solutions (1 pmol/µL) were directly infused at a flow rate of 5 µL/min. IM spectra were acquired in V-mode for 1 min using MassLynx, processed using DriftScope, and collision cross section (CCS) values calculated.

Table 1. Custom synthesized oligonucleotide sequences and molecular weights

Name	Sequence	Theoretical Molecular Weight (g/mol)
FLP	UCACUUUCAUAAUGCUGG	7126.2
Deamin_1	UUACUUUCAUAAUGCUGG	
Deamin_2	UCAUUUUCAUAAUGCUGG	7127.2
Deamin_3	UCACUUUUAUAAUGCUGG	
n-G_1	UCACUUUCAUAAUGCUGG	6706.9
n-G_2	UCACUUUCAUAAUGCUGG	
n-U_1	UCACUUUCAUAAUGCUGG	
n-U_2	UCACUUUCAUAAUGCUGG	
n-U_3	UCACUUUCAUAAUGCUGG	6732.0
n-U_4	UCACUUUCAUAAUGCUGG	
n-U_5	UCACUUUCAUAAUGCUGG	

Results and Discussion

Multiply charged deprotonated ions, $[M - xH]^{x-}$ where x indicates the number of protons lost, were formed by ESI for all oligonucleotides tested in this study. The most abundant charge states were $[M - 7H]^{7-}$ and $[M - 8H]^{8-}$. Each charge state was isolated, and the DT was measured repeatedly on three different days.

➢ *FLP and Deamination Products*

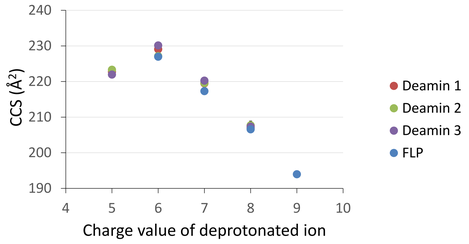

Figure 2. Plot of CCS *vs* charge state of the FLP and deamination products.

Three deamination products isobaric to the FLP were tested for their mobility. The CCS value is plotted against charge state (**Figure 2**).

- For $[M - 7H]^{7-}$, a difference in CCS between the FLP and the deamination products (Deamin 1 to 3) is observed with the FLP having a slightly smaller CCS.
- For $[M - 6H]^{6-}$, Deamin 2 has nearly identical CCS to FLP, which is slightly smaller than CCS of Deamin 1 and 3.
- An inflection point is observed at the -6 charge state, likely resulting from the molecule’s conformation change occurred at gas phase [2].

Additionally, the percent DT shift of a deamination product relative to the DT of the FLP (*i.e.*, % Relative DT shift) was calculated. The % Relative DT shift was compared to the average relative standard deviation (RSD) of the DT measured from 3 replicates of FLP (reference).

- The % Relative DT shift of all deamination products for $[M - 7H]^{7-}$ exceeds the RSD (%) of the FLP by over 1-fold, indicated by the yellow color coded for n-fold where n is between 1 and 2 **(Table 2)** .
- A % Relative DT shift value of a deamination product exceeding the RSD (%) of FLP (*i.e.*, n > 1) indicates a detected difference in mobility between the deamination product and the FLP.

Table 2. The RSD (%) of the FLP at different charge states of deprotonated ions and the % Relative DT shift (relative to FLP) of the deamination products.

	RSD (%) of DT		
Reference:	*-6*	*-7*	*-8*
FLP	0.79	0.64	0.66

	% Relative DT shift		
	-6	*-7*	*-8*
Deamin_1	0.71	0.86	0.08
Deamin_2	0.33	0.92	0.13
Deamin_3	1.10	1.09	0.07

n-fold: ≤ 1 | > 1 | > 2 | > 3

n = **% Relative DT shift / RSD (%) of DT of reference**

➢ *n-G Impurities*

Two isomeric impurities from a guanine nucleotide deletion at two different sites along the FLP sequence were tested for their mobility (**Figure 3**).

- The n-G impurities were indistinguishable by IM regardless of charge state.
- Similarly, an inflection point is observed at the -6 charge state.

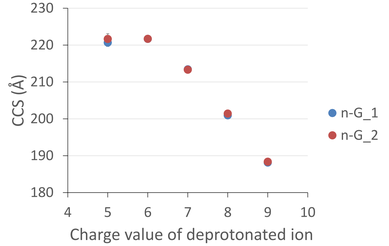

Figure 3. Plot of CCS *vs* charge state of the n-G impurities

➢ *n-U Impurities*

Five impurity isomers with one uracil nucleotide deletion at varying sites along the FLP sequence were tested for their mobility.

- For $[M - 7H]^{7-}$, the CCS of n-U_1 markedly differs from that of the remaining four isomers (**Figure 4**).
- The % Relative DT shift of the n-U_2 to 5 isomers (relative to n-U_1 as reference) exceeds the RSD (%) of n-U_1 by over 3-fold for $[M - 7H]^{7-}$, indicated by the red color coded for n-fold where n is > 3 **(Table 3)**.

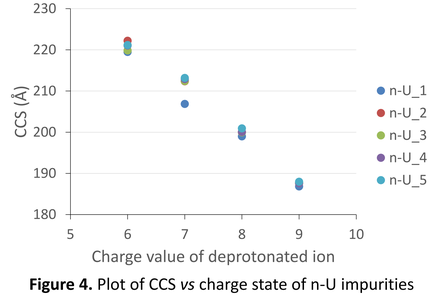

Figure 4. Plot of CCS *vs* charge state of n-U impurities

Table 3. The RSD (%) of n-U_1 at different charge states of deprotonated ions and the % Relative DT shift (relative to n-U_1) of the n-U_2 to 5 isomers.

	RSD (%) of DT			
Reference:	*-6*	*-7*	*-8*	*-9*
n-U_1	1.1	1.0	1.1	1.1

	% Relative DT shift			
	-6	*-7*	*-8*	*-9*
n-U_2	1.5	3.3	0.6	0.6
n-U_3	0.2	3.3	0.6	0.5
n-U_4	0.9	3.6	0.7	0.5
n-U_5	0.9	3.8	1.2	0.8

Note: color code same as Table 2.

➢ *Mixture of n-U Impurities*

Two mixtures of n-U impurity isomers were tested for their potential separation by mobility.

- For the mixture of n-U_1 and 5 isomers, two ion populations well-separated by mobility were observed for $[M - 7H]^{7-}$. Furthermore, the CCS values measured for the two ion populations well match with those measured for the two individual isomers when analyzed separately (**Figure 5**, left panel).
- In contrast, one ion population was observed for the mixture of n-U_2 to 4 isomers at all observed charge states (**Figure 5**, right panel).

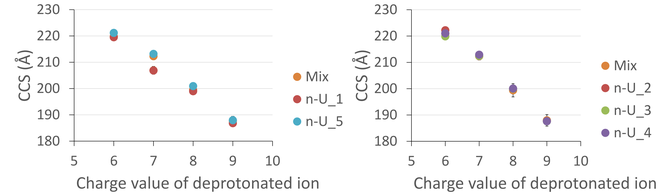

Figure 5. Plot of CCS *vs* charge state of equimolar mixtures of n-U_1 and 5 (left) and n-U_2 to 4 (right).

Conclusions

IM provides an additional dimension of separation based on molecular shape and mobility, complementary to LC and MS separation.

- IM analysis of the tested oligonucleotide molecules results in an inflection point, likely reflecting a gas-phase conformational change of the molecule.
- After passing the inflection point, the CCS decreases inversely with charge state.
- The difference in ion mobility between isomeric or structurally similar molecules is charge-state dependent.
- Single-pass cIM was able to differentiate between two n-U isomers whose conformation appears different at the -7 charge state. Future work will involve multi-pass cIM (HRIM) to potentially improve the resolving power.

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Disclaimer

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