Mass spectrometry-based structural analysis of oligonucleotide DNA duplexes

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INTRODUCTION

Oligonucleotides of different types (e.g. DNA, siRNA, RNAi, mRNA, ASO), lengths (from a 10-20 base pairs to full mRNAs) and higher-order structure (single strands, duplexes) have been increasingly studied due to their use in cell & gene therapies. This increased interest has also brought about a wider usage of mass spectrometry to characterize these types of molecules, owing to features such as high sensitivity, mass confirmation and sequencing capabilities (including modified oligonucleotides) and the ability to provide structural information (using fragmentation and ion mobility).

In this work, proof-of-principle data obtained for synthetic DNA duplexes illustrates how ion mobility - mass spectromtery measurements can be used to obtain structural information on oligonucleotide duplexes, analyzed by means of size-exclusion chromatography (SEC) in non-denaturing conditions, coupled to electrospray ionization (ESI).

Among key features shown are the ionization and transmission of solution duplexes into the gas phase with minimal perturbation of native structure, collision cross-section measurements or such ions, and comparisons between simplex mixing and a full annealing protocol with regards to their gas-phase structures.

METHODS

Synthetic DNA sense (SS) and antisense (AS) strands, with lengths ranging from 24 to 72mer each strand, were obtained from IDT (**Table 1**). Sequences were picked with at least 60% C/G content to facilitate formation of stable duplexes. A commercial dsiRNA duplex (25/27mer, already annealed) was also obtained and used to optimize settings for MS transmission of the duplexes.

The remaining sets of DNA SS and AS (24mer, 36mer, 48mer and 72mer) were individually resuspended in annealing buffer and either just mixed or mixed and subjected to an annealing protocol to see if any differences are observed.

Table 1. Identities and average masses of the duplex species studied.

Oligo	Single Strand Average Mass (Da)	Duplex Average Mass (Da)
dsiRNA SS (25mer)	7925.8	16527.0 (52mer)
dsiRNA AS (27mer)	8601.2	
ds24mer DNA SS	7408.8	14706.6 (48mer)
ds24mer DNA AS	7297.8	
ds36mer DNA SS	10948.2	22118.5 (72mer)
ds36mer DNA AS	11170.3	
ds48mer DNA SS	15132.8	29540.1 (96mer)
ds48mer DNA AS	14407.3	
ds72mer DNA SS	22593.6	44371.6 (144mer)
ds72mer DNA AS	21778.0	

To assess whether the protocol for preparation of duplexes from single strands had any impact on their resulting structures, sets of DNA SS and AS were individually resuspended at 200 pmol/µL each in annealing buffer (10 mM Tris.HCl, 50 mM NaCl, 1 mM EDTA, pH 7.5) and either just mixed, or mixed and subjected to an annealing protocol with heating and gradual cooling (heat to 95°C for 2 min, cool to 80°C over 15 min, then 65°C over 15 min, then 50°C over 15 min and finally to room temperature over 15 min for a total of 1 h).

Resulting samples (100 pmol/µL) were transferred to Waters QuanRecovery™ vials and analyzed using an ACQUITY™ Premier UPLC™ system coupled to a SELECT SERIES™ Cyclic™ IMS mass spectrometer.

Size-exclusion chromatography (SEC) under non-denaturing conditions was carried out on a ACQUITY Premier UPLC system with a TUV detector (260 nm). A BEH™ SEC 200 Å, 4.6 × 300 mm, 1.7 µm column was used (room temperature), with an isocratic flow (0.2 mL/min) of 50 mM ammonium acetate for 20 min. Loadings of 200 pmol were used, which correspond to 3-9 µg depending on species.

The Cyclic IMS (**Fig. 1**) was operated in ESI+ Mobility TOF mode (Single Pass), with typical settings known to work well for non-denaturing analysis of large biomolecules, and optimized using the dsiRNA 25/27mer standard to ensure no gas-phase dissociation into single strands was seen. CCS calibration was performed by direct infusion using a nanoESI source and coated glass capillaries. Poly-alanine, bovine serum albumin (BSA) and Concanavalin A (from jack bean) were used as calibrants, infused in 50 mM ammonium acetate at concentrations ranging from 5-15 µM.

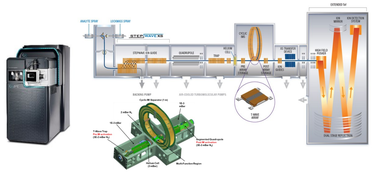

Figure 1. Schematics of the SELECT Series Cyclic IMS instrument.

Spreadsheets were used to process calibration data and allow conversion of mobility chromatogram drift time axes to N_2 CCS in Å^2.

RESULTS

A typical chromatogram and spectra obtained after optimizing MS transmission are shown on **Fig. 2** for the dsiRNA duplex.

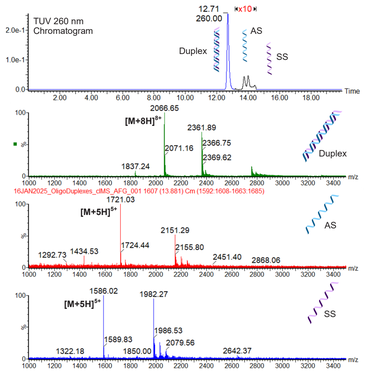

Figure 2. SEC chromatogram and mass spectra for the species observed in the dsiRNA duplex sample used to optimize MS conditions.

While the duplex species is prevalent in the chromatogram, some residual sense (SS) and antisense (AS) single strands are still seen and separated. Spectra show narrow charge state distributions consistent with transmission to the gas phase in non-denaturing conditions. No single strand peaks are seen in the spectrum of the duplex LC peak at 12.71 min.

Upon inspection, native SEC UV chromatograms for the other duplexes (**Fig. 3**). don't show any residual single strand peaks, demonstrating again prevalence of the duplex form. Moreover, no significant differences are seen when comparing between simple mixed and fully annealed samples.

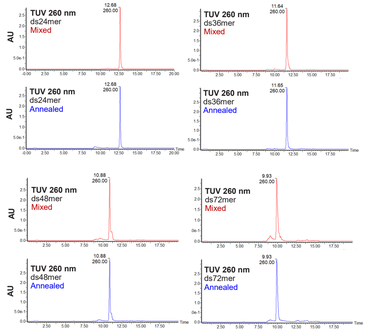

Figure 3. Non-denaturing SEC chromatograms (TUV 260 nm) for all the duplex species studied, prepared either with simple mixing or full annealing.

Spectra for the main duplex peaks are shown on **Fig. 4**. Apart from different charge state distributions which increase with duplex size, little to no difference is seen between mixing and annealing conditions.

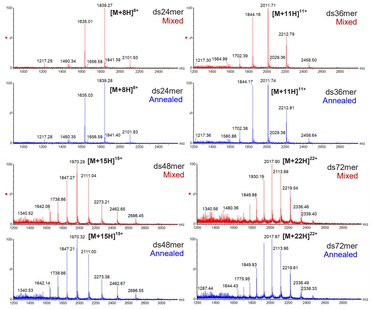

Figure 4. Mass spectra for duplex LC peaks observed for all duplex species studied.

Next, ion mobility arrival time distributions (ATDs) were investigated for the main charge states observed for each duplex species. **Fig. 5** below shows the CCS calibration curve obtained which was used to convert drift time (ms) to N^2 CCS in Å.

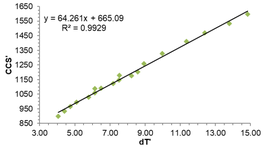

Figure 5. CCS calibration curve obtained.

For the ds24mer (**Fig. 6**) with charge states ranging from +7 to +10, more compact conformations are seen for the +7 and +8 charge states as expected around 1400-1700 Å^2. ATDs overlay almost perfectly, showing no significant differences between mixing and annealing.

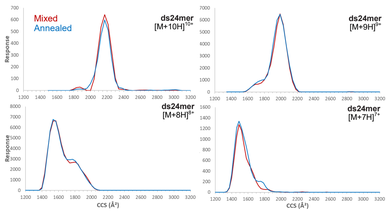

Figure 6. Ion mobility ATDs for the dsDNA 24mer duplex.

Some differences appear for the ds36mer (**Fig. 7**), for which the most compact conformation is seen around 2000 Å^2. This conformation is clearly more prevalent for the annealed sample versus the mixed sample, as shown for example for the +10 charge state.

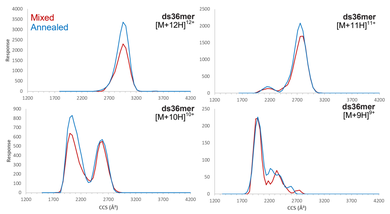

Figure 7. Ion mobility ATDs for the dsDNA 36mer duplex.

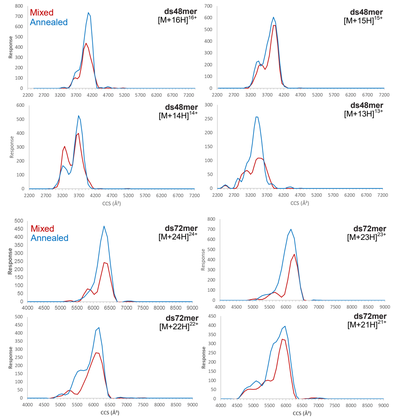

Figure 8. Ion mobility ATDs for the dsDNA 48mer (top) and 72mer (bottom) duplexes.

A similar trend is seen for the ds48mer and ds72mer (**Fig. 8**). Both show more complex ATD profiles illustrating a wider population of conformations, however the annealed condition is always shown to favor more compact conformations (e.g. ~3370 Å^2 for the ds48mer +13 and between 5500-6000 Å^2 for the ds72mer +21 to +23). The more compact conformations are also seen for the mixed condition, however with lower response.

CONCLUSION

We demonstrated the use of IM-MS to probe the gas-phase structure of DNA duplexes of different sizes, generated by either simple mixing or full annealing protocols.

An approach based on non-denaturing SEC makes the entire experiment more amenable to routine conditions employed in the industry for the separation of biomolecules. Interestingly, no differences were seen in the LC dimension, while IM-MS allowed estimating molecule sizes based on CCS and provided evidence that the annealing protocol favors more compact structures (likely closer to native solution structures) versus simple mixing.

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