Separation of Intact Protein Isoforms with Differential Mobility

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ABSTRACT

Combining size exclusion chromatography (SEC) and differential ion mobility (DMS) to improve separation of conformational isoform in MS analysis.

INTRODUCTION

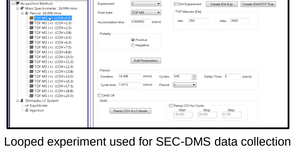
Native electrospray offers the ability to maintain structural characteristic of the proteins and protein complex prior to MS analysis [1]. Much of the early work performed in this area relied on direct infusion at very low flow rate (<1µL/min) of protein solutions that mimic biological condition that favor non-covalent interaction (water @ pH 7). Using nanospray conditions enabled significant increase sensitivity under these conditions. With the advent of the coupling of conventional mobility with MS detection, protein and protein complex could be interrogated based on conformational differences. The ability to measure difference in conformation with mobility offers new means to understand complex system and their interaction in biological system.

Size exclusion chromatography (SEC) can be used to separate protein complex in solution based on their molecular weight. Unlike other commonly used LC medium used (reversed phase), SEC typically offer lower resolution when small differences in isoforms need to be identified. SEC also offers the ability to perform separation under conditions that are close to native conditions. The coupling of SEC and differential mobility (DMS) could offer means to interrogate proteins under condition that approach biological conditions and offer insight into differential conformation by relying on separation of species in the gas phase, prior to MS analysis. Here we present the coupling of SEC-DMS-MS-MS analysis proteins under conditions that are 'native-like' and some of the considerations during analysis.

MATERIALS AND METHODS

All proteins and peptide standards were acquired from SIGMA and used without any further purification. All solutions were prepared in 20mM ammonium formate adjusted to pH 7. LC was performed using a Shimadzu Nexera UFLC system operated at a flow rate of 350µL/min with a 4.6x150mm Yara 3u SEC-3000 (Phenomenex). Isocratic conditions were used for elution: 20mM NH4 formate at pH 7 (90%) and acetonitrile 0.1% formic acid (10%)

All samples were analysed using a TripleTOF® 6600 system equipped with a SelexION® device (DMS system) using a Turbo V™ source. Under SEC conditions, the source was operated at a temperature of 500ºC with Gas 1 and Gas 2 at 40 and 70psi, respectively. All DMS measurements were performed at separation voltage (SV) of 4000V, with cell temperature (DT) set to 150ºC and resolving gas (DMR) set to 10psi (low).



Looped experiment used for SEC-DMS data collection

DMS Separation of Cytochrome-C and Ubiquitin

In order to characterize the experimental conditions that may have impact on detection of proteins species and their conformers, we used simpler systems to assess some of the critical parameters that would need to be considered. Cytochrome-C has been well studied under a variety of MS systems with and without mobility [2-4] and conditions that can affect solution conformation have been well documented. Like many proteins, cytochrome-c can be denatured in solution by altering the pH from neutral to acidic, or by addition of organic solvent. At pH 7, cytochrome-c is expected to predominantly be present as a compact form, whereas addition of organic solvent will generate a bi-model MS response that is frequently associated with mixtures of protein conformation that range from compact form to more open structure. The presence of multiple conformation generated under these solution condition can revels multiple CoV optima or broader CoV range for given charge state, as depicted in Figure 1. Similar observation were made with ubiquitin, another model protein frequently used in MS-mobility experiments [5,6].

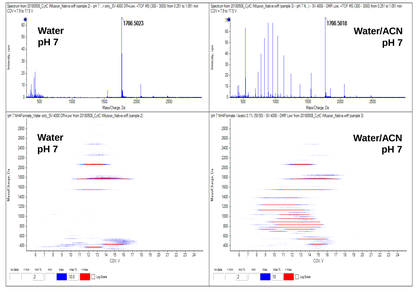


Figure 1

Cytochome-C infused at 8µL/min in 20mM ammonium formate adjusted to pH 7 in water and water/acetonitrile (50/50) solutions. Mass spectra were acquired with DMS turned off. The DMS data was collected at SV=4000 and DR=10psi (LOW). Higher charge state exhibit multiple CoV optima (m/z 600-800) whereas lower charge states tend to exhibit broad CoV range of signal (unresolved)

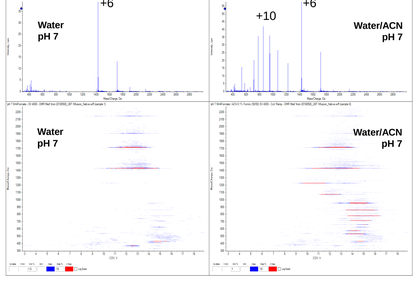


Figure 2

Ubiquitin infused at 8µL/min in 20mM ammonium formate adjusted to pH 7 in water and water/acetonitrile (50/50) solutions. Mass spectra were acquired with DMS turned off. The DMS data was collected at SV=4000 and DR=25psi (MED). Higher charge state exhibit multiple CoV optima with the z=+10 charge state having baseline separation (2 distinct optima)

SEC-DMS Separation of Cytochrome-C and Ubiquitin

SEC was performed under partial denaturation conditions (10% of ACN:Formic (99.9:0.1) and 90% 20mM NH_4-formate pH7) to generate species that would exhibit differences in CoV optima. DMS data was collected in 'loop-experiment' covering a CoV range from 1 to 20V, using step of 1V and resolution gas set to 10psi (LOW). Under these conditions, complete separation of cytochrome-c, ubiquitin and exenatide peptides was achieved. Looking at the CoV distribution, a systematic shift towards higher CoV value was observed with increasing charge state. Similar to infusion data, multiple CoV optima were observe for specific charge sate, suggesting differential conformation could be maintained and detected under higher LC flow rate conditions (which typically require higher source temperatures). Focusing on the +12 charge state cytochrome-c (m/z 1030.5), it is possible to observe 2 distinct CoV optima at 8.2 and 12.5. Similar values were observed under infusion conditions when acetonitrile was used for this charge state as well as some of the other charge states that could be resolved. However, the dominant lower charge state (z=7) only showed 2 CoV optima under LC conditions, where up to 3 optima were observed under infusion conditions. This may suggest that the addition heat supplied by the source to handle the increase in solvent flow may impart additional denaturation of the protein.

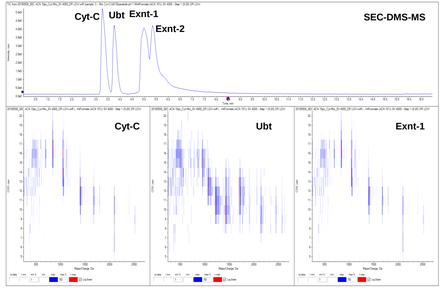


Figure 3

SEC-DMS for mixture of cytochome-c, ubiquitin, and exenatide peptides. Isocratic conditions were used (see experimental section). The DMS data was collected at SV=4000 and DR=10psi (LOW) and CoV was stepped from +5 to +20 in 1 volt increments.

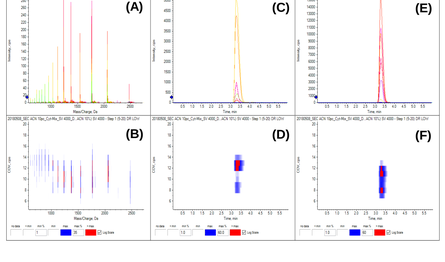


Figure 4

SEC-DMS for mixture of cytochome-c, ubiquitin, and exenatide peptides. Average overlaid spectra for all CoV value (A) and heat map (B). XIC for m/z 1031 (C) and 1547 (E), representing charge state +12 and +7, respectively. LC-CoV heat map for 1031 (D) and 1547 (F).

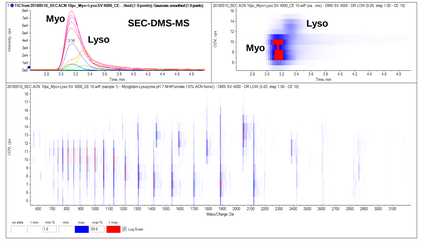


Figure 5

SEC-DMS for mixture of myoglobin and lysozyme. Isocratic conditions were used (see experimental section). The DMS data was collected at SV=4000 and DR=10psi (LOW) and CoV was stepped from +5 to +20 in 1 volt increments. Overlay TIC (top-left), LC-CoV heat map (top-right) and heat map m/z-CoV (bottom).

Similar observation were made with myoglobin and lysozyme (Figure 5). In this particular case, co-elution of the 2 proteins lead to mixed MS spectra. Though little overlap existed between these proteins, the DMS provided additional separation that simplified the spectra. It is also important to note in this case that lysozyme systematically showed higher CoV values. Lysozyme contains 4 disulfide bonds that assist in the stabilization of the proteins, which could suggest that more compact structure should exhibit higher CoV values.

CONCLUSIONS AND FUTUR WORK

- DMS can assist in reducing the mass spectral complexity when SEC is used.
- DMS can reveal the presence of isoforms under SEC separation
- Care must be taken to account for all sources of denaturation that can occur (mobile phase, temperature, etc.) to ensure consistency in measurements
- Future work will focus on impact of temperature and as well as combining this approach with gas phase H/D exchange to gain further insight into protein structure and isomer differentiation.

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