HIGHER SENSITIVITY, DATA QUALITY AND THROUGHPUT FOR HYDROGEN DEUTERIUM EXCHANGE EXPERIMENTS WITH EXTENDED ION MOBILITY SEPARATION

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INTRODUCTION

Characterization of higher order structure in proteins and protein complexes is integral for understanding the function and mechanisms of protein action and is essential to the evaluation and development of therapeutic biomolecules. Hydrogen-deuterium exchange (HDX) has become a standard request for characterization of proteins and protein complexes and in addition to providing dynamic structural insight, its use in high-throughput epitope screening has also gained favor.

For complex systems, performance improvements in the LC-MS system, higher sensitivity and/or peak capacity support richer and higher quality datasets, which in turn can provide more detailed information to the biological question at hand. Utilization of shorter chromatographic gradients yields higher deuterium retention but results in higher spectral complexity, which can be a challenge for *in-silico* database searches. Consequently, the quality of experimental data is typically a balance between protein size and chromatographic length. As the trend over recent years has been the study of larger protein complexes, often with 10s of subunits, spectral complexity is increasingly a challenge.

The new SELECT SERIES™ Cyclic™ IMS, with enhanced ion mobility and mass resolution, offers improved peak capacity to address these challenges. Here, the performance of the Cyclic IMS system for hydrogen deuterium exchange is demonstrated relative to the SYNAPT™ G2-*Si* and SYNAPT™ XS, for sample limited applications, and for higher throughput data collection.

METHODS

Experiments were performed on a LEAP HDX-2 Automation robot coupled to an ACQUITY™ M-Class HDX system with online pepsin digestion (Enzymate™ column (Waters)) and a SELECT Series Cyclic IMS mass spectrometer, using variable loads of rabbit glycogen phosphorylase B (P00489, PhosB) as a model protein.

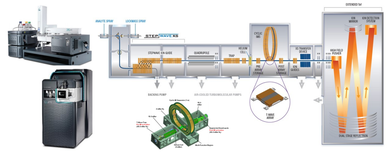

Figure 1. *LEAP HDX-2 Automation/ACQUITY M-Class HDX system and SELECT Series Cyclic IMS system with schematics.*

HDX and LC conditions are summarized below. Intact protein was mixed at a 1:20 ratio with 10 mM, pH 7.0 phosphate buffer and quenched with a 1:1 dilution into 100 mM, pH 2.4 phosphate buffer at 0 °C prior to injection into the LC system.

SAMPLE

Glycogen Phosphorylase B (Rabbit), #P00489
Lyophilized solid reconstituted to make stock solution at ~32.0 µM/ monomer

SOLUTIONS

Equilibration (E) buffer: 10 mM potassium phosphate in H_2O, pH 7.0
Labeling (L) Buffer: 10 mM potassium phosphate in D_2O), pH 6.6 (pD 7.0)
Quench (Q) buffer: 100 mM potassium phosphate in H_2O, pH 2.40
Pepsin Column Wash: 1.5 M guanidine hydrochloride, 4.0% acetonitrile, 0.8% formic acid, pH 2.40

INCUBATION, QUENCH AND INJECTION VOLUMES

Protein sample volume: 3.0 µL
Equilibration buffer volume: 57 µL
Transfer volume: 50.0 µL
Quench volume: 50.0 µL (quench aliquot kept at 0.5ºC)
Injection volume: 95.0 µL (50.0 µL Loop)

LC CONDITIONS

Pepsin Column	Enzymate BEH Pepsin Column 2.1 × 30 mm, 5 µm
Digestion Temp.	15ºC
Trapping Column	VanGuard BEH C18, 2.1 × 5 mm, 1.7 µm
Analytical Column	ACQUITY UPLC BEH C18 1.0 × 100 mm, 1.7 µm
HDX Temp.	0.2ºC
µBSM A:	H_2O with 0.1% FA
µBSM B:	ACN with 0.1% FA
ASM A1:	H_2O with 0.05% FA
ASM B1:	100 fmol/µL Leu-Enk/GFP, H_2O/ACN 75:25 0.1% FA
LEAP Weak Wash	H_2O with 0.1% FA
LEAP Strong Wash	95:5 H_2O/ACN with 1% FA
Seal Wash:	H_2O/MeOH 90:10

LC GRADIENTS

Either an original (12 min run-time, 7-min analytical gradient) or a truncated LC gradient (8 min run-time, 3–min analytical gradient) were evaluated to assess higher throughput capability.

Analytical 7-min Gradient

Time (min)	Flow Rate (µL/min)	%A	%B
Initial	**40.0**	**95.0**	**5.0**
7.0	40.0	65.0	35.0
7.5	40.0	15.0	85.0
9.0	40.0	15.0	85.0
9.1	40.0	95.0	5.0
12.0	40.0	95.0	5.0

Analytical 3-min Gradient

Time (min)	Flow Rate (µL/min)	%A	%B
Initial	**60.0**	**95.0**	**5.0**
3.0	60.0	65.0	35.0
3.2	60.0	15.0	85.0
3.8	60.0	15.0	85.0
4.0	60.0	95.0	5.0
8.0	60.0	95.0	5.0

Trapping 3-min

Time (min)	ASM A1 Flow Rate (µL/min)
Initial	75.0
1.0	200.0
2.2	200.0
2.5	75.0
3.0	75.0

MS METHODS

Peptides were analyzed using an HDMS^E (DIA) method using either single or double pass cyclic ion mobility. For purposes of comparison, Synapt G2-Si and Synapt XS data were acquired using previously described settings [1] under the same experimental conditions. Relevant Cyclic IMS parameters are shown below:

Ionization Mode:	Positive
Acquisition Range:	*m/z* 50-2000
Capillary Voltage:	2.8 kV
Transfer CE:	20-29 V
Cone Voltage:	30 V
StepWave1 Offset	8 V
StepWave2 Offset	12 V
Number of Passes	1 or 2 (Separate 5 or 25 ms)
TW Static Height	25 V
Sideways TW Velocity	375 m/s
ADC Pushes per Bin	1

Processing

Data was processed using ProteinLynx Global Server (PLGS), 3.0.3 and DynamX 3.0

For reference (time zero) data, three replicates were processed and searched by PLGS using LE/HE thresholds set at 150 and 30.

Peptide ion accounting files were imported into DynamX in the form of .CSV outputs.

Peptides identified by PLGS were filtered at this stage for both quality of identification and reproducibility. This was done by applying restrictive parameters to the imported peptide lists [2] as shown on Figure 2.

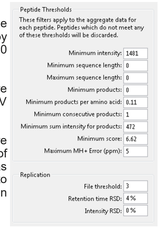

Figure 2. *DynamX filtering parameters used, as described by Sørensen et al [2].*

RESULTS

Initial experiments were undertaken to benchmark the performance of the Cyclic IMS system relative the SYNAPT G2-Si and the recently released SYNAPT XS. Data was collected using HDMS^E with 32 µM stock PhosB solution and with identical Stepwave™ ion guide settings between the SYNAPT XS and Cyclic IMS. Peptide mapping experiments of Phosphorylase B were used to evaluate the performance of the system in terms of peptide identifications in complex mixtures.

Triplicate injections were independently processed in PLGS 3.0.3 using standard processing parameters and the resulting peptide files imported into DynamX and filtered as described previously. The number of identified peptides and resultant sequence coverage obtained on the three instruments is reported in Figure 3.

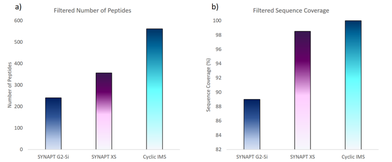

Figure 3. *Number of identified peptic peptides and sequence coverage following filtering[2] for Phosphorylase B using the SYNAPT G2-Si, SYNAPT XS, and Cyclic IMS mass spectrometers.*

Improved performance was observed on the SYNAPT XS and Cyclic IMS relative to the SYNAPT G2-Si in terms of sequence coverage. Approximately 240, 360, and 560 filtered peptides were identified using the SYNAPT G2-Si, SYNAPT XS, and Cyclic IMS, respectively (Figure 3a). The addition of the StepWave XS device in the SYNAPT XS and Cyclic IMS yields improved sensitivity, which likely contributes to the improvement to the number of identifications. The Cyclic IMS system has several other attributes, including the scalable IMS resolution, XS transfer device, and dual gain ADC detection system that likely contribute to its improved performance over the SYNAPT XS.

Sequence coverages for the three instruments were 89, 98.5, and 100%. The small difference in coverage for the SYNAPT XS and Cyclic IMS is a direct result of the coverage already being exceptionally high; proteins with longer sequences are likely to experience a greater gain in coverage.

Consequently, loading and gradient truncation experiments were undertaken for the Cyclic IMS to evaluate HDX peptide mapping performance for lower loadings and with shorter chromatographic separations.

LOADINGS

A challenge of HDX experiments is that peptides resulting from low frequency pepsin cleavage often need to be monitored in order to access specific regions of the protein sequence. Consequently, high loads are often used experimentally to obtain better signal for these peptides. As a result, highly abundant peptides often appear with saturated and distorted isotope ratios and often contain artifact peaks resulting from overloading the detector.

The Cyclic IMS system features a dual gain ADC detector that improves the dynamic range of detection for highly abundant ions and the ADC improves the linear response of the detector at high ion currents. It was hypothesized that this feature would ameliorate the ion saturation often seen in HDX experiments and the data obtained supports this. In Figure 4, isotope distributions are shown for two peptide ions on the SYNAPT XS (bottom) and Cyclic IMS (middle), demonstrating the improved accuracy of the isotope measurements for high intensity ions. The theoretical isotope distribution for both ions is shown on the top.

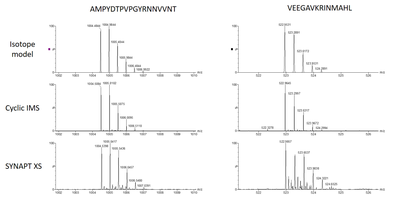

Figure 4. *Isotope distributions for peptides AMPYDTPVPGYRNNVVNT and VEEGAVKRINMAHL obtained on the SYNAPT XS (bottom) and Cyclic IMS (middle). The theoretical isotope distribution for each peptide is shown on the top for reference.*

Peptide identifications and sequence coverage for PhosB were examined using 8, 16, and 32 µM stock solutions to evaluate HDX performance on the Cyclic IMS under more sample-limited situations. Considering the labeling (1:20) and quench dilutions (1:1) described and a 50 µL loop, this corresponds to 10, 20, and 40 pmol injections. Figure 5 summarizes the results of this experiment.

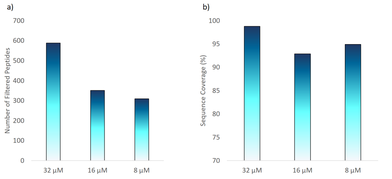

Figure 5. *Number of identified peptic peptides and sequence coverage following filtering for Phosphorylase B using 32, 16, and 8 µM stock solutions for online HDX peptide mapping with the Cyclic IMS, corresponding to 40, 20 and 10 fmol loads, respectively.*

Data was collected in HDMS^E mode using the same conditions described previously. The data was processed using PLGS 3.0.3 but with modified Peptide3D algorithm parameters used to specifically account for the slightly wider arrival time distributions observed with longer mobility separations. This had the effect of increasing the number of peptides in the 32 µM stock solution injections to 588.

As expected, lower loads yielded fewer peptide identifications; however, it should be noted that the same thresholds were used for all three conditions. It may be possible to recover some peptide identifications by lowering the thresholds for the lower load data. Nevertheless, better than 90% filtered sequence coverage was obtained for the 16 and 8 µM solutions, 93% and 95%, respectively, indicating that good performance HDX peptide mapping can be achieved with lower concentration stock solutions, allowing for study of more sample limited proteins.

TRUNCATED GRADIENT

Considering the Cyclic IMS's variable mobility resolution and separation capabilities, experiments were performed to determine if the LC gradient could be truncated for high-throughput purposes, whilst maintaining similar performance by using the enhanced mobility separation to differentiate and individually detect among a complex mix of peptides with a higher level of co-elution.

The flow rate was increased to 60 µL and a modified gradient with 3-min effect analytical separation (8-min total run) was employed (see Methods). On the Cyclic IMS , HDMS^E acquisitions were configured with either a single or double pass of ions on the Cyclic device. Example chromatograms are shown in Figure 6.

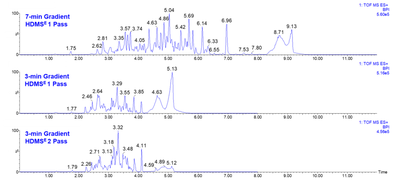

Figure 6. *Example MS BPI chromatograms for the original and truncated gradient experiments.*

To provide some insight into the added complexity of the truncated gradient, Figure 7 shows extracted ion chromatograms (XICs) and example spectra obtained for three specific peptides of the PhosB peptide digest, both for the original 7-min and truncated 3-min gradient.

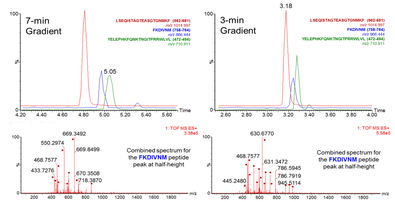

Figure 7. *Examples of added complexity in terms of chromatographic separation and spectral complexity between the original 7-min and truncated 3-min gradient.*

We can see that not only the three peptides exemplified show a higher degree of coelution from a reduced LC separation space, but the spectral complexity is also greater within the retention time range of a single peptide. This can lead to overlapping peptide isotope patterns and difficulty to distinguish between them in order to measure deuterium uptake. This is a situation were enhanced ion mobility separation, especially with variable resolution as on the Cyclic IMS, plays an important role in allowing better distinction between peptide ions within a more complex space.

In terms of overall filtered number of identified peptides and sequence coverage for Phosphorylase B, the impact of employing a truncated gradient with either 1- or 2-Pass Cyclic mobility separation is shown on Figure 8.

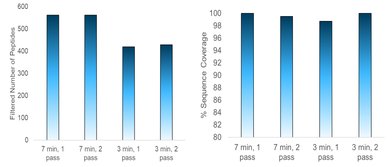

Figure 8. *Comparison of filtered number of peptides (left) and sequence coverage (right) for PhosB between the original 7-min and truncated 3-min gradient, using either 1- or 2-Pass Cyclic IMS separation.*

While using a 2-Pass method with the original 7-min gradient provides the highest number of filtered identified peptides, the numbers observed for the 3-min truncated gradient, though lower, are not heavily impacted. This can be confirmed by the sequence coverage percentages, which are very similar between all experiments, indicating that key peptides providing sequence coverage and information are still detected even with the truncated gradient.

CONCLUSION

We have previously shown a significant improvement in the quality of HDX data obtained from the SYNAPT XS compared to the older generation, SYNAPT G2-Si. Here, the new SELECT SERIES Cyclic IMS is demonstrated to offer the highest level of performance and has a profound impact on the quality of data obtainable for hydrogen-deuterium exchange experiments. Improved mass and ion mobility resolution and sensitivity result in 30% more peptide identifications than on the SYNAPT XS while the dual gain detector allows for more accurate measurement of isotope distributions for highly abundant peptides.

The improved sensitivity of the system permits a 4x load reduction while retaining better than 95% sequence coverage. By using a truncated gradient with less than half the analytical gradient time, similar sequence coverage could still be obtained by employing Cyclic mobility separation. This demonstrable improvement to peak capacity and sensitivity is expected to afford use of truncated chromatographic gradients, enhancing daily throughput and reducing the per injection cost of the system.

REFERENCES

[1] Morrison, L., Anderson, M., Quinn, C. Enhanced Performance of the SYNAPT XS and Its Impact on Hydrogen Deuterium Exchange Mass Spectrometry (HDX MS) Data Quality. Waters Technical Note #720006870 (https://www.waters.com/webassets/cms/library/docs/720006870en.pdf)

[2] Sørensen, L., Salbo, R. Optimized Workflow for Selecting Peptides for HDX-MS Data Analyses. *J. Am. Soc. Mass Spectrom.* **2018**, *29*, 2278–2281. https://doi.org/10.1007/s13361-018-2056-1

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