LC separation optimization for XL-MS Analysis

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Abstract

Purpose: To optimize LC separation for XL-MS workflows on Thermo Scientific™ Orbitrap™ Ascend™ and Thermo Scientific™ Astral™ mass spectrometers.

Methods: Amine-reactive crosslinkers DizSEC[1] (2,5-Dioxopyrrolidin-1-yl (2-(3-methyl-3H-diazirin-3-yl)ethyl)carbamate), DSSO (disuccinimidyl sulfoxide) and DSBU (disuccinimidyl dibutyric urea) were used to crosslink standard proteins and *E.coli* cell lysate. Separation was achieved using Thermo Scientific™ Vanquish™ Neo UHPLC system with various gradients and Thermo Scientific™ EASY-Spray™ PepMap™ columns, µPAC™ Neo HPLC Columns or IonOpticks Aurora Ultimate™ columns. Peptides were detected on Thermo Scientific™ Orbitrap™ Ascend™ or Astral™ mass spectrometers and data were analyzed using XlinkX node in Thermo Scientific™ Proteome Discoverer™ v3.0 and Scout v1.4.14[2].

Results: PepMap columns perform the best for the detection of crosslinked peptides. For the crosslinker DizSEC, we developed a new MS2-MS3 method to improve the accuracy of XL identifications.

Introduction

Cross-linking mass spectrometry (XL-MS) has grown dramatically as a key workflow for elucidating protein higher-order structure and mapping protein-protein or protein-nucleic acid interaction networks on a proteome-wide scale. However, significant LC separation challenges remain due to sample complexity and low abundance of XL peptides. In this work, we evaluated XL-MS workflows with various newly released columns including the Thermo Scientific™ µPAC™ Neo HPLC Columns and IonOpticks Aurora Ultimate™ columns. We also optimized the MS method for new cleavable crosslinker DizSEC on Orbitrap Ascend mass spectrometer.

Materials and methods

Sample Preparation

Three amine-reactive crosslinkers: DizSEC, DSSO and DSBU were used to crosslink standard proteins and *E.coli* cell lysate. Crosslinked samples were digested and spiked into Hela digest in various ratios.

Liquid Chromatography and Mass Spectrometry

Samples were separated by reverse phase-HPLC on a Thermo Scientific™ Vanquish™ Neo system using different columns including EASY-Spray™ PepMap™ RSLC C18 column, µPAC™ Neo HPLC Column or IonOpticks Aurora Ultimate™ column. Detailed specifications are listed in Table 1. Peptides were eluted over a 60 min 6-50% gradient (A: water, 0.1% formic acid; B: 80% acetonitrile, 0.1% formic acid) at 300 nL/min flow rate. The crosslinked samples were analyzed on Thermo Scientific™ Orbitrap™ Ascend™ or Thermo Scientific™ Astral™ mass spectrometers in DDA modes. Specific LC and MS settings are shown in Tables 2 and 5.

Data Analysis

The acquired spectra were analyzed using Thermo Scientific™ Proteome Discoverer™ 3.0 software: the XlinkX node with the open or MS2 search option for crosslinked peptides and SEQUEST™ HT search engine for unmodified, looplinks and monolinks peptides. Data were also analyzed using Scout v1.4.14. Carbamidomethylation (+57.021 Da) was used as a static modification for cysteine. Different crosslinked mass modifications for lysine were used as variable modifications for lysine or N-terminus in addition to methionine oxidation (+15.996 Da). Data were searched against a protein database containing 10 proteins including bovine serum albumin (P02769) or human serum albumin (P02768) with a human or *E.coli* database. The false discovery rate (FDR) was set to 1% at CSM and cross-link levels.

Results

Comparison of different columns

We compared the performance of three reverse phase columns using BSA DSSO samples (30 fmol) spiked into 100ng of Hela digest. The column specifications are listed below. Several aspects were evaluated including peak widths, carryover, identified crosslinks, looplinks and monolinks.

Table 1. Specifications of reverse phase columns used for XL-MS workflows.

	 PepMap	µPac	 Aurora
Dimensions	75 µm × 25 cm	50 cm	75 µm × 25 cm
Particle size or Pillar size	2 µm	2.5 µm	1.7 µm
Max. Pressure	800 bar	450 bar	1700 bar

Table 2. LC gradient and MS acquisition parameter settings for BSA DSSO spiked in Hela Experiments.

Retention [min]	Flow [nL/min]	Aurora	µPac	PepMap
0.1	300	6.0	6.0	4.0
40.1	300	20.0	25.0	25.0
60.1	300	35.0	40.0	40.0
61.1	300	50.0	50.0	99.0

Settings	Ascend	OT-Astral
MS1 scan	Orbitrap	Orbitrap
Resolution	60K	180K
Normalized AGC Target (%)	100%	500%
Max. injection time (ms)	123	5
Charge state	3-8	3-8
MS mass range, m/z	380-1400	400-1400
RF lens	60	40
MS2 scan	Orbitrap	Astral
Resolution	30K	Standard
Normalized AGC Target (%)	200%	500%
Max. injection time (ms)	70	20
Charge states	3-8	3-8
First mass (m/z)	120	150
Intensity threshold	$5e^4$	$1e^4$
Isolation width	1.6	1.6
NCE (%)	SCE 20, 25, 30	32(z=3)+28(z>3)
Top speed	3s	1s

Figure 1. LC profiles of BSA DSSO spiked in Hela digest using different columns and a 60 min gradient.

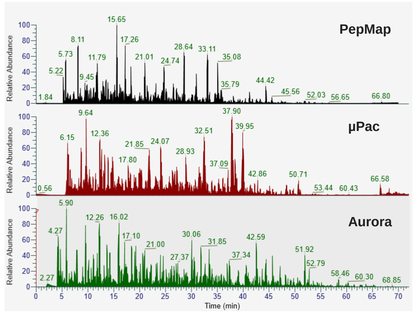


Figure 2. Venn diagram showing the overlap of identified BSA DSSO crosslinks spiked into HeLa digest using different columns and OT Ascend.

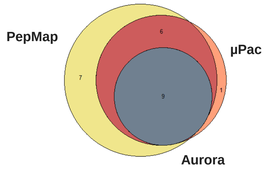
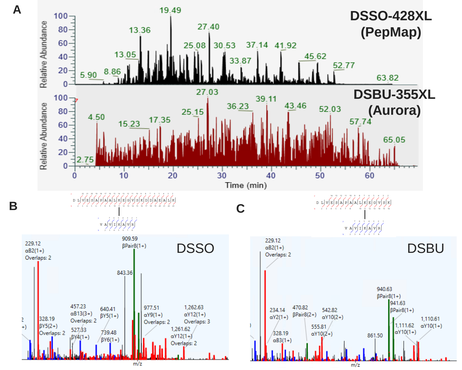


Table 3. Summary of column performance and search results.

	PepMap	µPac	Aurora
Baseline LC peak width	0.33	0.57	0.33
Carryover	1%	0.5%	2.5%
HeLa Proteins	2545	2797	3255
PSMs	6986	8149	9646
XLs	22	16	9
CSMs	43	32	14
Looplinks	11	8	7
Monolinks	38	36	19

Figure 3. (A) LC profiles of *E.coli* crosslinked samples using a 60 min gradient on OT-Astral. Identifications DSSO (B) or DSBU (C) crosslink Lys96-Lys71 in 50S ribosomal protein L12 using Scout.



Methods for DizSEC crosslinked samples

DisSEC is a novel MS-cleavable heterobifunctional crosslinker that connects Lys with any amino acid in proteins. Collisional energy is crucial for cleavable crosslinkers. To improve accuracy in identification, we developed a MS2-MS3 method for DizSEC crosslinked peptides. Optimized parameters are listed below.

Figure 4. Structure and reactions of MS-cleavable heterobifunctional photo-cross-linkers DizSEC.

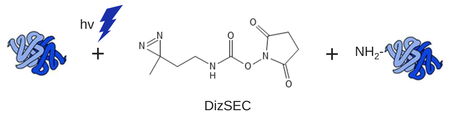
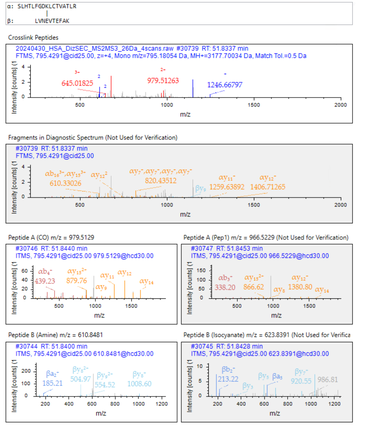


Table 5. Orbitrap Ascend MS acquisition parameter settings for DizSEC MS2-MS3 method.

Settings	MS2	MS3
Detector	Orbitrap	Ion trap
Resolution/Scan Rate	30K	Rapid
Normalized AGC Target (%)	100%	200%
Max. injection time (ms)	59	80
Charge states	3-8	2-6
Intensity threshold	$1e^4$	NA
Isolation width	1.6	2.5
NCE (%)	CID 25	HCD 30
Top speed/scans	5s	4 scans
Delta Mass (Da)	25.9942	

Figure 5. Example of an HSA DizSEC crosslinker identified by MS2- MS3 method.



Conclusions

- Increased numbers of HeLa proteins were identified using µPAC or Aurora columns compared to the PepMap column but fewer crosslinked peptides.
- Significant carryover from the Aurora column was observed for standard cross-linked protein samples.
- An optimized MS2-MS3 method for DizSEC crosslinker was developed to reduce false positives in XL identifications.

References

1. Faustino AM, Sharma P, Manriquez-Sandoval E, Yadav D, Fried SD. Progress toward Proteome-Wide Photo-Cross-Linking to Enable Residue-Level Visualization of Protein Structures and Networks In Vivo. *Anal Chem.* 2023 Jul 18;95(28):10670-10685.

2. Milan Avila Clasen, Max Ruwolt, Louise U. Kurt, Fabio C Gozzo, Shuai Wang, Tao Chen, Paulo C Carvalho, Diogo Borges Lima, Fan Liu. Proteome-scale recombinant standards and a robust high-speed search engine to advance cross-linking MS-based interactomics. *bioRxiv* 2023.11.30.569448

Conflict of interest

YH, ER, LF, RB and RV are employees of Thermo Fisher Scientific.

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