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Cross-linking Mass Spectrometry

# Optimized XL-MS workflows for heterobifunctional crosslinkers SDA and DizSEC

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## Abstract

**Purpose:** To optimize XL-MS workflows for heterobifunctional photoactivatable -crosslinkers SDA and DizSEC.

**Methods:** Heterobifunctional photoactivatable -crosslinkers SDA (succinimidyl 4,4'-azipentanoate), sulfo-SDA (sulfosuccinimidyl 4,4'-azipentanoate) and DizSEC (2,5-Dioxopyrrolidin-1-yl (2-(3-methyl-3H-diazirin-3-yl)ethyl)carbamate) were used to crosslink standard proteins. Separation was achieved using Thermo Scientific™ Vanquish™ Neo LC system with a 60 min gradient using Thermo Scientific™ EASY-Spray™ PepMap™ Neo column. Following separation, the peptides were detected on Thermo Scientific™ Orbitrap Ascend™ or Exploris 480 ™ mass spectrometers, and data were analyzed using XlinkX node in Thermo Scientific™ Proteome Discoverer™ v3.2 software and xiSEARCH/xiFDR. Identified XLs were visualized using the XMAS plug-in for ChimeraX.

**Results:** With optimized MS2 and MS2-MS3 methods, SDA and DizSEC crosslinked peptides can be identified with high confidence.

## 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. Photoactivatable, heterobifunctional crosslinkers like SDA can link lysine residues with any residue, providing detailed information for protein structures, but also pose challenges due to lack of MS-cleavability and increased search space during data analysis. Recently, a new cleavable crosslinker, DizSEC, was developed to overcome this issue. DizSEC links the same residues as SDA, but DizSEC crosslinked peptides can generate unique peptide pairs in MS/MS to facilitate data analysis.

In this work, we optimized XL-MS workflows for SDA and DizSEC crosslinked samples and developed a new MS2-MS3 method to improve DizSEC XL identifications.

## Materials and methods

### Sample preparation

Heterobifunctional photoactivatable -crosslinkers SDA (succinimidyl 4,4'-azipentanoate), sulfo-SDA (sulfosuccinimidyl 4,4'-azipentanoate) and DizSEC (2,5-Dioxopyrrolidin-1-yl (2-(3-methyl-3H-diazirin-3-yl)ethyl)carbamate) were used to crosslink standard proteins.

### Liquid chromatography and mass spectrometry

Samples were separated by reverse phase-HPLC on a Vanquish Neo system using Thermo Scientific™ EASY-Spray™ PepMap™ RSLC C18 column. 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™ Exploris™ 480 mass spectrometers in DDA modes. Specific LC and MS settings are shown in Tables 1, Figure 2, and Table 3.

### Data analysis

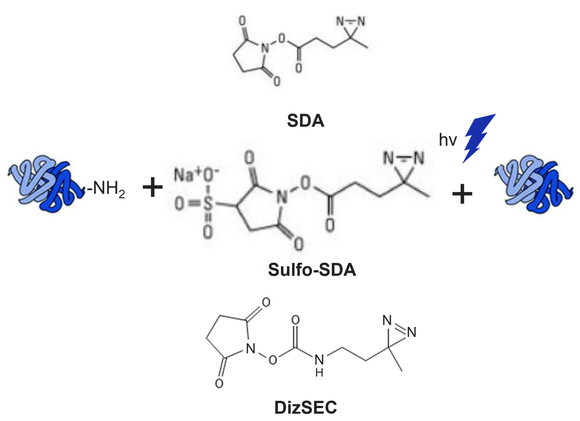
The acquired spectra were analyzed using Proteome Discoverer 3.2 software: the XlinkX node with the noncleavable, MS2 or MS2-MS3 search option for crosslinked peptides and SEQUEST™ HT search engine for unmodified, looplinks and monolinks peptides. Detailed settings for DizSEC is shown in Figure 5. Data were also analyzed using xiSEARCH/xiFDR. 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 human serum albumin (P02768) or yeast enolase (P00924). The false discovery rate (FDR) was set to 1% at CSM and cross-link levels.

## Results

### Comparison of three crosslinkers

We compared the performance of three heterobifunctional photoactivatable -crosslinkers SDA , sulfo-SDA and DizSEC with standard proteins. The structures of the crosslinkers are shown in Figure 1.

**Figure 1. Structure and reactions of three heterobifunctional photo-cross-linkers SDA, LCSDA and DizSEC.**




## Results

**Table 1. Orbitrap Exploris and Ascend MS acquisition parameter settings for SDA and DizSEC MS2 methods.**

| Settings | Exploris | Ascend |
|---|---|---|
| **MS1 scan** | Orbitrap | Orbitrap |
| Resolution | 120K | 240K |
| Normalized AGC Target (%) | 300% | 150% |
| Max. injection time (ms) | 20 | 100 |
| Charge state | 3-8 | 3-8 |
| MS mass range, m/z | 375-1600 | 380-1400 |
| RF lens | 50 | 60 |
| **MS2 scan** | Orbitrap | Orbitrap |
| Resolution | 45k | 60K |
| Normalized AGC Target (%) | 200% | 500% |
| Max. injection time (ms) | 105 | 123 |
| Charge states | 3-8 | 3-6 (prioritizing +4 to +6) |
| First mass (m/z) | 150 | 150 |
| Intensity threshold | $1e^4$ | $2.5e^4$ |
| Isolation width | 1.6 | 1.4 |
| NCE (%) | SCE 20,25, 30 | SCE 20, 25, 30 |
| Top speed | 3s | 5s |

**Figure 2. Orbitrap Ascend MS acquisition methods for SDA and DizSEC crosslinked peptides.**

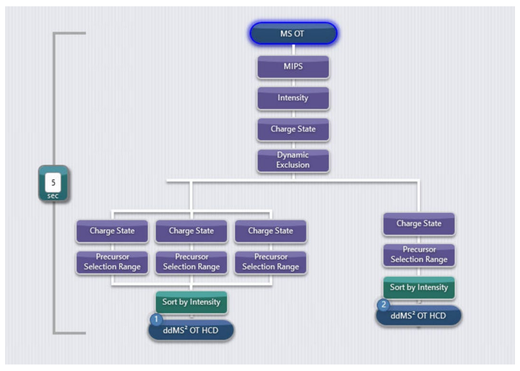



**Table 2. Summary of XlinkX non-cleavable search results from three crosslinkers on OT Exploris 480.**

| | | Looplinks | CSMs | Crosslinks |
|---|---|---|---|---|
| HSA | SDA | 148 | 200 | 113 |
| | Sulfo-SDA | 128 | 171 | 99 |
| | DizSEC | 117 | 164 | 108 |
| Enolase Dimer | SDA | 75 | 107 | 53 |
| | Sulfo-SDA | 75 | 93 | 60 |
| | DizSEC | 51 | 52 | 17 |

**Figure 3. Venn diagram showing the overlap of identified enolase crosslinks on OT Exploris 480.**

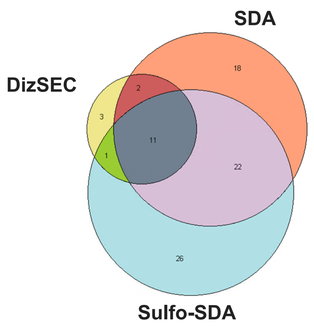
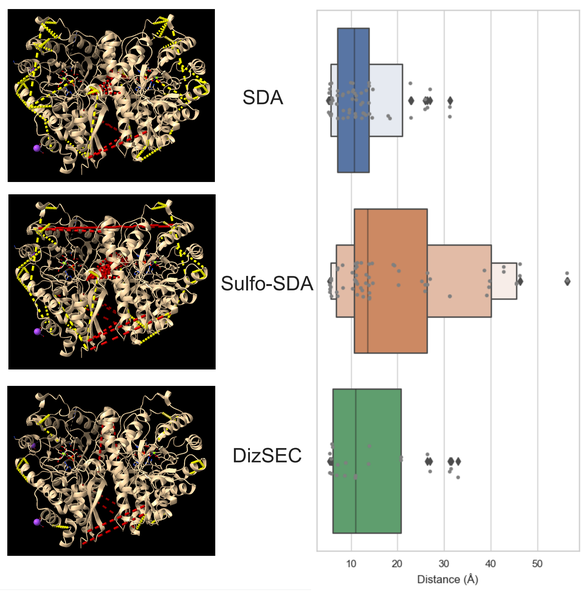



**Figure 4. Intra (yellow) and inter (red) crosslinks identified in the enolase dimer structure with three crosslinkers and their distance distributions.**




## Results

### Methods for DizSEC crosslinked samples

DisSEC is a novel MS-cleavable heterobifunctional crosslinker that connects Lys with any amino acid in proteins and forms Ureas. Ureas are doubly fissile under HCD/CID (across each N-carbonyl bond) and generate two distinct sets of fragment ions (as shown in Figure 5). Collisional energy is crucial for cleavable crosslinkers. To improve accuracy in identification, we developed a MS2-MS3 method for DizSEC crosslinked peptides to compared with the MS2 method. Optimized parameters are listed below.

**Figure 5. Structure and MS fragmentation of DizSEC crosslinked peptides.**

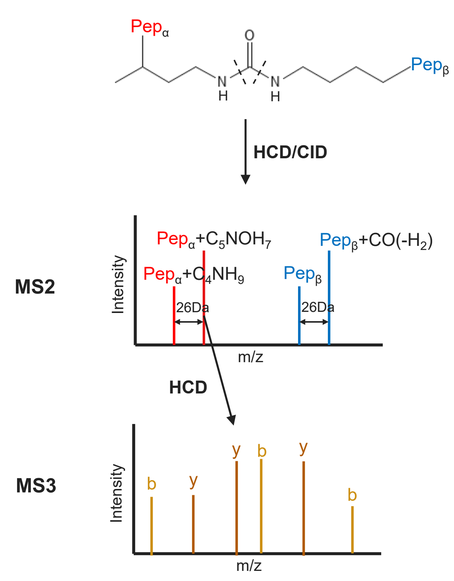
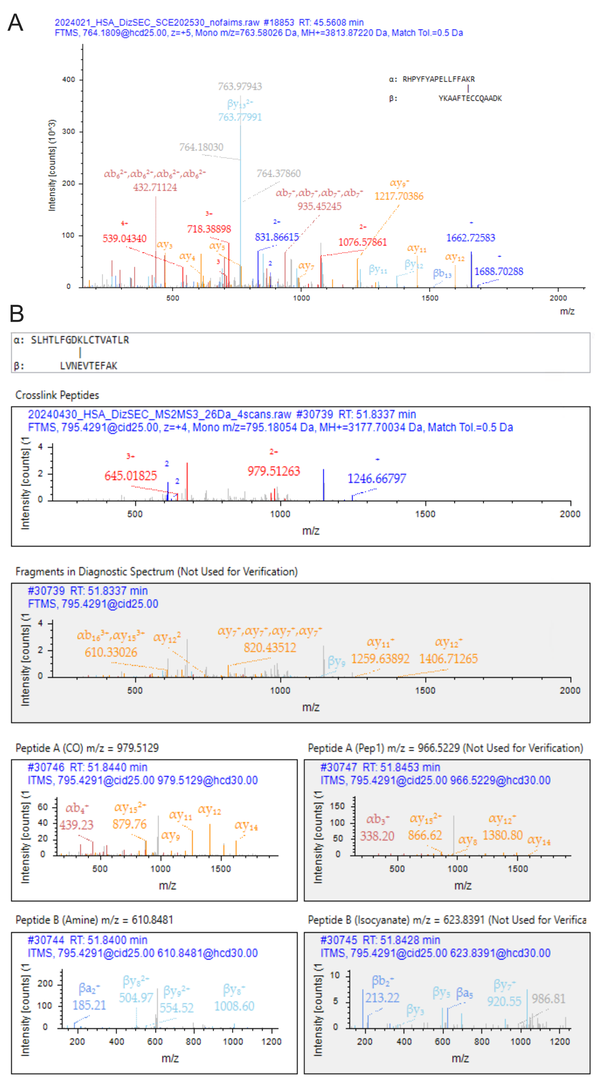



**Table 3. Orbitrap Ascend MS acquisition parameter settings for DizSEC MS2-MS3 method.**

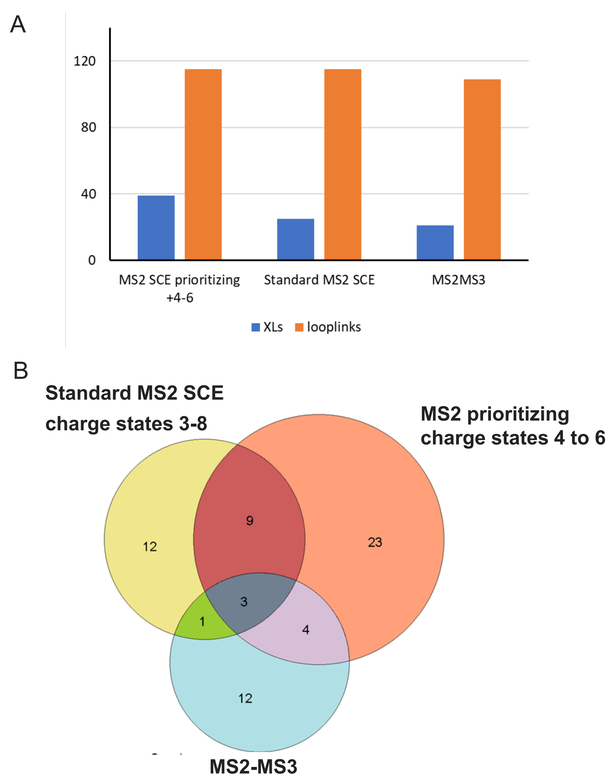
| Settings | MS2 | MS3 |
|---|---|---|
| Detector | Orbitrap | Ion trap |
| Resolution/Scan Rate | 30K | Rapid |
| Normalized AGC Target (%) | 100% | 200% |
| Max. injection time (ms) | 59 | 80 |
| Charge states | 3-8 | 2-6 |
| Intensity threshold | $1e^4$ | NA |
| Isolation width | 1.6 | 2.5 |
| NCE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

**Figure 6. Examples of HSA DizSEC crosslinkers identified by (A) MS2 method and (B) MS2- MS3 method.**




## Results

**Figure 7. (A) HSA DizSEC crosslinks and looplinks identified using XlinkX MS2 search in different MS methods. (B) Venn diagram showing the overlap of identified HSA crosslinks on OT Ascend.**




## Conclusions

- Three different SDA crosslinkers provided a similar number of crosslinked peptides for monomeric proteins.
- SDA and sulfo-SDA outperformed DizSEC for multimeric proteins probably due to the length of the linker.
- Increased numbers of SDA and DizSEC crosslinked peptides were identified using the instrument method prioritizing high charge states 4-6.
- An optimized MS2-MS3 method for DizSEC crosslinker was developed to reduce false positives in XL identifications.

## References

1. Tanaka Y, Kohler JJ. Photoactivatable crosslinking sugars for capturing glycoprotein interactions. J Am Chem Soc. 2008 Mar 19;130(11):3278-9.
2. Mendes ML, Fischer L, Chen ZA, Barbon M, O'Reilly FJ, Giese SH, Bohlke-Schneider M, Belsom A, Dau T, Combe CW, Graham M, Eisele MR, Baumeister W, Speck C, Rappsilber J. An integrated workflow for crosslinking mass spectrometry. Mol Syst Biol. 2019 Sep;15(9):e8994.
3. Fischer L, Rappsilber J. Quirks of Error Estimation in Cross-Linking/Mass Spectrometry. Anal Chem. 2017 Apr 4;89(7):3829-3833.
4. 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.
5. Lagerwaard IM, Albanese P, Jankevics A, Scheltema RA. Xlink Mapping and AnalySis (XMAS)—Smooth Integrative Modeling in ChimeraX. bioRxiv: 2022.04.21.489026.

## Conflict of interest

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

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