Optimized XL-MS workflows for membrane protein analysis

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ABSTRACT

Purpose: To develop end to end XL-MS workflows for membrane proteins using the membrane permeable crosslinkers.

Methods: Membrane permeable crosslinker tBuPhoX (tert-butyl disuccinimidyl phenyl phosphonate) and DSS (disuccinimidyl suberate) were used to crosslink membrane proteins *S. enterica* WbaP. Separation of digested peptides was achieved using Vanquish™ Neo LC system with a 60 min gradient. Following separation, the peptides were detected on Thermo Scientific™ Orbitrap Eclipse™ and data were analyzed using Thermo Scientific™ Proteome Discoverer™ 3.0 software and XlinkX 3.0 node. Identified XLs were visualized using the XMAS plug-in for ChimeraX.

Results: With the optimized crosslinking workflow, three inter-XLs between subunits were identified with high confidence providing strong evidence for the dimer structure of membrane protein WbaP.

INTRODUCTION

Cross-linking mass spectrometry (XL-MS) has grown dramatically into a routinely utilized strategy for characterizing protein higher-order structure and mapping protein-protein interaction networks on a proteome-wide scale. However, the XL-MS analysis of membrane proteins is still a significant challenge due to their hydrophobic properties as well as the lipid-rich environment. In this work, we optimized XL-MS workflows for membrane proteins in SMALPs or detergents with membrane permeable, phospho-enrichable crosslinker- tBuPhoX[1-2].

MATERIALS AND METHODS

Sample Preparation

Membrane protein S. enterica WbaP in SMALPs was prepared in 20 mM HEPES buffer pH 7.0. DSS or tBuPhoX solutions in DMSO were used to crosslink 600 µg/mL WbaP at room temperature for 1 hr. After crosslinking reaction, the sample was either prepared to SDS-PAGE gel analysis or buffer exchanged into 1% DDM. Gel bands were sliced and digested with either trypsin or pepsin. The sample in 1% DDM was incubated on ice for 30 min. SMALP was removed by adding MgCl2 (50 mM stock) to a final concentration of 4 mM, incubating at 4 ºC for 1 hr, and then centrifuged at 21,000 ×g at 4 ºC for 1 hr. The supernatants were diluted 1:1 0.1% SDS, reduced, alkylated and digested with either trypsin (1:20 ratio (enzyme to protein) or pepsin (1:50 ratio (enzyme to protein). tBuPhoX sample digests (60 µg) were enriched using the TiO2 Phosphopeptide Enrichment Tips according to the manufacturer's instructions.

Liquid Chromatography and Mass Spectrometry

Samples were separated by reverse phase-HPLC using a Thermo Scientific™ Vanquish™ Neo system connected to a Thermo Scientific™ EASY-Spray™ PepMap™ 75 um x 25 cm column over a 60 min 3-65% 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 the Orbitrap Eclipse™ Tribrid™ mass spectrometer with Instrument Control Software version 4.0. Specific LC and MS settings are shown in Table 1.

Data Analysis

The acquired spectra were analyzed using Proteome Discoverer 3.0 software using the XlinkX node 3.0 for crosslinked peptides and SEQUEST™ HT search engine for unmodified, looplinks and monolinks peptides. Data were searched against the sequence of N-term dual strep tagged S. enterica WbaP with 1% FDR criteria for crosslink spectra matches. For data acquired using the non-cleavable crosslinkers, DSS or PhoX, a search option in XlinkX-NonCleavable-open was used. Crosslinks sites in WbaP dimer were visualized in XMAS plug in for ChimeraX[3].

RESULTS

Figure 1. The cross-linking workflow for membrane protein in SMALP

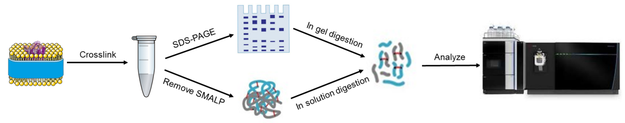
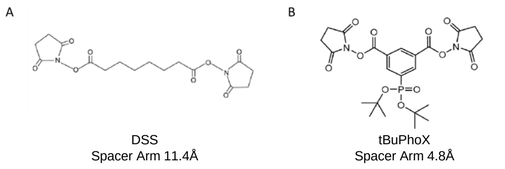


Figure 2. Structures of crosslinkers used in the study. (A) DSS, (B) tBuPhoX



DSS
Spacer Arm 11.4Å

tBuPhoX
Spacer Arm 4.8Å

In-solution digestion provides better sequence coverage and XL identifications

In this experiment, we tested two different workflows (in-gel digestion vs in-solution digestion). The results are summarized in Table 1. In both workflows, we are able to achieve sequence coverages more than 80%. Importantly, SMALP was successfully removed during the in-solution workflow for the downstream LS-MS analysis. Detailed information is shown in Figure 1 including the sequence coverage and modifications. The only region that was not identified is part of the transmembrane domain of the protein.

Table 1. Summary of search results from in-gel digestion and in-solution digestion.

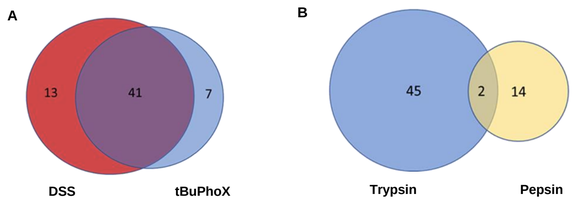
Method	Sequence Coverage	XLs	CSMs	XL MS2 scans
In gel	81.96%	32	62	62
In solution	93.86%	61	224	270

Figure 3. The sequence coverage of S. enterica WbaP (93.86%) from in-solution workflow.

Crosslinking reagents and digestion enzymes

We evaluated two membrane permeable crosslinkers (DSS vs tBuPhoX) and two digestion enzymes (trypsin vs pepsin). As shown in Figure 4, majority of identified XLs were the same between DSS and tBuPhoX. More DSS XLs were identified mainly due to the more flexible and longer linker of DSS. In contrast, trypsin and pepsin each provide unique XLs with only two XLs overlapped.

Figure 4. Venn diagrams showing the overlap of crosslinking sites identified using (A) two crosslinkers, (B) two enzyme digestion.



Inter-XLs were identified to confirm the dimer structure

Identified XLs were mapped onto the predicted dimer structure of *S. enterica* WbaP using the XMAS plug-in for ChimeraX. Four potential inter-XLs were observed with high confidence (Figure 5). Furthermore, their distances were compared in both intra- and inter- situations and three of them are only valid when the linkage is the between the subunits (Table 2). Take together, this result provides unambiguous evidence for the dimer structure and provides information for the interface between subunits (Figure 6).

Table 2. Inter crosslinks identified in WbaP dimer.

Residue 1	Residue 2	Distance –intra (Å)	Distance –inter (Å)
Lys81	Lys273	**28.4**	**20.7**
Lys148	Lys273	43.2	**20.8**
Lys232	Lys273	46.3	**13.0**
Lys222	Lys422	42.2	**26.6**

* Number in green indicates that the distance is within the maximum distance of the crosslinker

Figure 5. MS/MS spectrum of the inter crosslink Lys148-Lys273

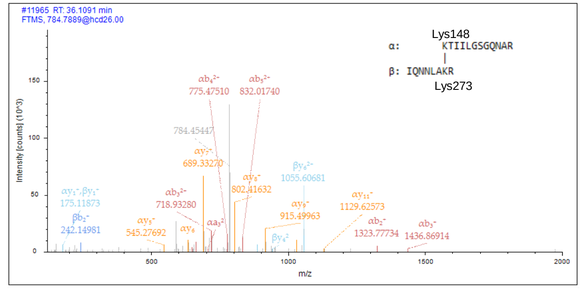
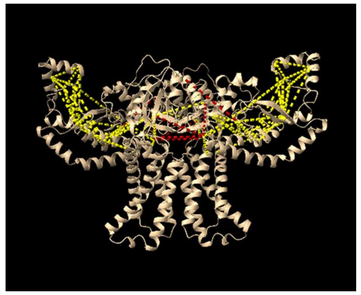


Figure 6. Intra (yellow) and inter (red) crosslinks identified in WbaP dimer structure.



CONCLUSIONS

- End to end XL-workflows for membrane proteins in SMALP were developed and evaluated using two membrane permeable crosslinkers DSS and tBuPhoX.
- The in-solution workflow enables a better sequence coverage and more XL identifications for membrane proteins .
- With the optimized crosslinking workflow, three inter-XLs between subunits were identified with high confidence confirming the dimer structure of membrane protein WbaP.

REFERENCES

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TRADEMARKS/LICENSING

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