Optimized Sample Preparation for Phospho-Enrichable Crosslinkers

LEIGH FOSTER[1]; Amarjeet Flora[1]; Premchendar Nandhikonda[1]; Rosa Viner[2]; Chris Etienne[1]; Ryan Bomgarden[1]

[1]Thermo Fisher Scientific, Rockford, IL; [2]Thermo Fisher Scientific, San Jose, CA

ABSTRACT

Purpose: To optimize DSPP and TBDSPP crosslinking conditions, phosphatase treatment, protein and peptide-level cleanup, acid deprotection, phospho enrichment, and adapt Thermo Scientific™ EasyPep™ sample prep chemistry for crosslinked peptide compatibility.

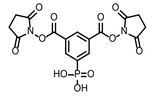
Methods: DSPP (Disuccinimidyl Phenyl Phosphonic Acid, PhoX) and TBDSPP (tert-Butyl Disuccinimidyl Phenyl Phosphonate, tBu-PhoX) were used to crosslink samples. Samples were prepared and separated by a Thermo Scientific™ Dionex™ UltiMate™ 3000 Nano LC system and detected on the Thermo Scientific™ Orbitrap Eclipse™ mass spectrometer. Data were analyzed using Thermo Scientific™ Proteome Discoverer™ software.

Results: Optimized crosslinked peptide workflow increased our identification of crosslinked peptides by 5-10x compared to traditional crosslinking workflows with DSS and DSSO in complex samples.

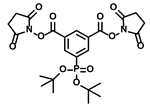
INTRODUCTION

Cross-linking mass spectrometry is a powerful method to determine protein-protein interactions and has been applied to protein complexes and intact cells to analyze interactions on a global scale. However, crosslinking suffers from low identification rates with typical yields of cross-linked peptides <1% of total peptides. This results in very few or no crosslinked peptide identifications by MS if the sample complexity is greater than a few hundred proteins. Both traditional, non-cleavable, and MS-cleavable crosslinkers can be used for the identification of protein-protein interaction sites; but enrichable crosslinkers are advantageous because they can be used to identify low abundant crosslinked peptides. Phospho-enrichable crosslinkers are ideal due to their high specificity of enrichment and simple, robust protocols. Here, we optimized two phospho-enrichable crosslinker workflows for DSPP and TBDSPP using a Fe-NTA magnetic agarose resin for enrichment.

DSPP



TBDSPP



MATERIALS AND METHODS

Sample Preparation for Bovine Serum Albumin (BSA) Crosslinking with DSPP

BSA samples were resuspended in PBS, pH 7.0, before crosslinking with 40x molar excess of crosslinker, then quenched with 15x crosslinker concentration of ammonium bicarbonate. 10kDa PES MWCO filters were used for buffer exchange into 50mM TEAB before reduction, alkylation, and digestion. Pierce Peptide Desalting Spin Columns were used for peptide level cleanup before enrichment with Fe-NTA magnetic agarose beads. Peptides were quantitated with Pierce™ Quantitative Fluorometric Peptide Assay before LC-MS analysis.

Sample Preparation for HeLa Cells Crosslinking in-vivo with TBDSPP

HeLa S3 cells were cultured in sMEM media supplemented with 10%FBS, 1X Glutamax, and 1% Pen/Strep. Cells were treated with Nocodazole at 0.1µg/ml for 24 hours. HeLa S3 harvested cells were resuspended in PBS pH 7.0 for crosslinking at 2.0-2.5mM, then quenched with 30-45mM ammonium bicarbonate before lysis in 0.1% SDS, 50mM TEAB, quantitation with BCA assay and alkaline phosphatase treatment. Acetone precipitation or protein aggregation capture (PAC) were used to remove excess crosslinker before reduction, alkylation, and digestion with trypsin/LysC between 2-18 hours. Samples were deprotected with TFA followed by peptide-level cleanup on tC18 resin. Fe-NTA magnetic beads were used for phosphopeptide enrichment before LCMS analysis.

LC-MS Acquisition and Data Analysis

Samples were separated using an UltiMate 3000 Nano LC system: BSA samples used a 15cm C18 Thermo Scientific™ EASY-Spray™ column with an acetonitrile gradient from 3% to 28% over 50 minutes at a flow rate of 300nL/min, and HeLa samples used a 50cm column with a gradient of 3% to 25% over 85 minutes and 25% to 40% over 40 mins. Samples were acquired on a Thermo Scientifc™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer, Orbitrap Eclipse Tribrid mass spectrometer, or a Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer. Data were analyzed with Proteome Discoverer 2.3 software and XlinkX node with a minimum crosslink confidence score of 40.

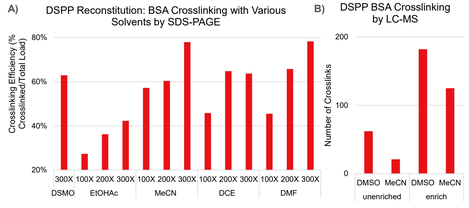
RESULTS

DSPP Crosslinking Workflow Optimization for Purified Proteins

Figure 1. DSPP Crosslinking Workflow

Crosslinking with DSPP and TBDSPP requires the following sample preparation steps: solubilization and introduction of crosslinker to sample for reaction, quenching, and protein-level cleanup of excess, unreacted crosslinker, reduction, alkylation, and digestion, peptide-level clean up, and phosphoenrichment before LC-MS analysis.

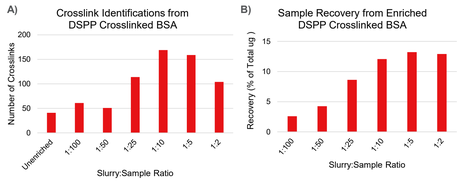
Figure 2. Increasing Crosslinking Identifications with Alternate Reconstitution Methods



DSPP is not readily soluble in DMSO; therefore, multiple solvents were tested for solubility with DSPP, followed by evaporation in a speed vac and reconstitution in DMSO. DSPP crosslinking is most efficient when solubilized in MeCN, dried down, and reconstituted in DMSO as shown for different molar excess ratios of crosslinker to protein.

A) Equivalent crosslinking was achieved with acetonitrile (MeCN) solubilization, evaporation, and reconstitution in DMSO and 1/3 crosslinker amount versus DMSO alone (analyzed by SDS-PAGE with Coomassie Blue Stain). B) MeCN alone was less suitable for crosslinking versus MeCN, evaporation, and DMSO reconstitution before crosslinking and LC-MS analysis.

Figure 3. Fe-NTA Magnetic Beads for Phosphoenrichment of DSPP Crosslinked Samples

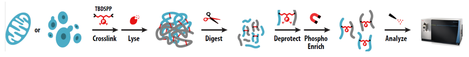


Clean DSPP crosslinked peptides can be directly applied to Fe-NTA magnetic beads for phosphoenrichment. A1:10 ratio of bead slurry to sample was determined to be sufficient for maximum crosslink identifications and sample recovery.

A) The proper ratio of Fe-NTA magnetic bead slurry was determined by titration of beads with a fixed amount of sample (500ug) to achieve the maximum number of crosslink identifications. B) Sample recovery was assessed using Pierce Quantitative Fluorometric Peptide Assay to determine enrichment yield.

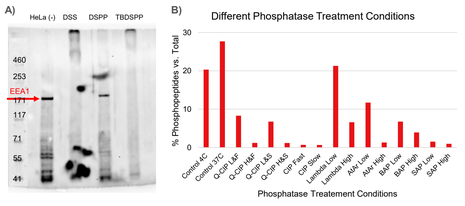
TBDSPP Crosslinking Workflow Optimization for in-vivo Structural Applications

Figure 4. TBDSPP Crosslinking Workflow



For *in vivo* crosslinking and sample preparation, additional steps are required. TBDSPP has the same reactivity and functionality as DSPP but has tert-Butyl "protective groups" which allow the crosslinker to permeate the membranes of cells for *in vivo* crosslinking applications. Acid deprotection of the tert-butyl groups is required before phosphoenrichment. Furthermore, treatment with phosphatase may be necessary to decrease competitive binding from endogenous phosphopeptides during enrichment.

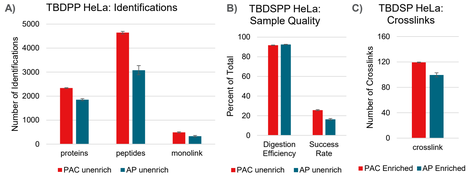
Figure 5. Demonstrating Membrane Permeability and Preparing Complex Matrix Samples



A) Western using 10µg of HeLa cell lysates with or without crosslinking using DSS, DSPP, and TBDSPP. Membrane permeability for different crosslinkers was assessed by an increase in molecular weight for an intracellular target, EEA1. B) Seven different phosphatases were tested in Nocodazole-treated HeLa using 2 different protocols each. Lysates were enriched with Fe-NTA magnetic beads to determine the number of phosphopeptides remaining after treatment.

DSS and TBDSPP demonstrate an increase in the molecular weight of EEA1 above 460kDa, suggesting membrane permeability, whereas lysate without crosslinking and lysate crosslinked with DSPP contain EEA1 at the anticipated molecular weight (~170kDa). CIP (Pierce Alkaline Phosphatase) was demonstrated to have the best removal of phospho groups on peptides.

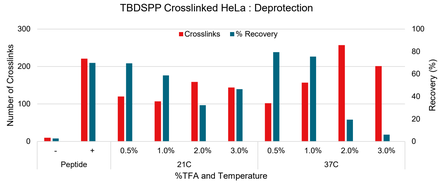
Figure 6. Improved Crosslinked Sample Clean-up with Protein Aggregation Capture



A) Protein Aggregation Capture (PAC) is a sample-prep technique which utilizes high-organic formulations to crash out and remove proteins from contaminants on a carboxylate-modified magnetic particle support following reduction and alkylation. PAC protocols resulted in more identifications in crosslinked samples versus acetone precipitation. B) Digestion efficiency in PAC workflows was equivalent to that achieved with acetone precipitation, whereas success rates were significantly improved (n=3). C) Crosslink identifications are improved in PAC protocols versus acetone precipitation after enrichment,

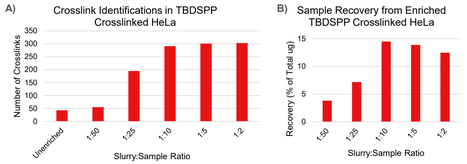
Better results were obtained with PAC versus acetone precipitation, which could be due to the more efficient removal of excess crosslinker which competes for binding on Fe-NTA magnetic beads.

Figure 7. Deprotection of Phosphonate in TBDSPP Before Phosphoenrichment



For peptide-level deprotection, 2.5% TFA at 37ºC for 1 hour resulted in the highest number of crosslink peptide identifications (notated as "+", with no deprotection notated as "-"). Deprotection was attempted at the protein level with increasing amounts of heat and TFA applied to crosslinked HeLa samples for 1 hour. Equivalent deprotection to that observed at the peptide level could be achieved using 2-3% TFA at 37º. However, using higher amounts of TFA at the protein level resulted in significant degradation of the samples which resulted in lower crosslinked peptide identifications.

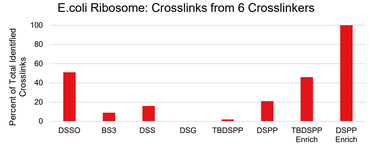
Figure 8. Fe-NTA Magnetic Beads for Phosphoenrichment of TBDSPP Crosslinked Samples



A) The optimized ratio of Fe-NTA magnetic bead slurry to crosslinked sample was determined by titration of beads with a fixed amount of sample (250µg) to achieve the maximum number of crosslink identifications. B) Sample recovery from phosphoenrichment was also assessed using Fluorometric Peptide Assay to determine the yield of peptides.

A 1:10 ratio of bead slurry to sample was determined to be sufficient for maximum crosslink identifications and sample recovery.

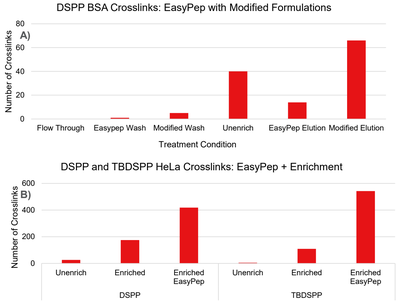
Figure 9. Crosslinker Benchmarking in E. coli Ribosomes



New England Biolabs *E. coli* ribosomes were crosslinked in PBS 7.0-7.5 with DSSO, BS3, DSS, DSG, DSPP, or TBDSPP at 2mM before sample preparation using optimized methods. Unenriched, DSPP and TBDSPP perform similarly to other non-cleavable crosslinkers. However, upon enrichment, ≥5x crosslinks were identified from the same samples, producing equivalent or greater numbers of crosslinks when compared to MS cleavable crosslinkers, such as DSSO.

EasyPep Chemistry and Compatibility with Phosphoenrichable Crosslinked Samples

Figure 10. DSPP and TBDSPP Crosslinking Compatibility with EasyPep Sample Preparation Workflows in HeLa Cell for Improved Single-Fraction Acquisition



A) EasyPep peptide cleanup buffers were modified for increased ionic strength for more stringent wash conditions while selectively retaining crosslinks for subsequent elution. B) Final protocol using EasyPep with modified TBDSPP deprotection, wash and elution formulations results in a significant increase in the number of crosslink identifications observed versus enrichment alone in a single-shot acquisition of a crosslinked HeLa cell fration.

Originally, crosslinked samples were incompatible with EasyPep chemistry. However, after modification of EasyPep buffer formulations, this chemistry can be used to decrease the amount of time and steps associated with the crosslinking protocol while simultaneously increasing crosslink identifications in single-shot fractions of complex samples, such as *in vivo* cell crosslinking.

CONCLUSIONS

- Protein-level cleanup of crosslinked samples is critical for excellent phosphoenrichment and maximum crosslink identifications
- Specialized workflow considerations for *in vivo* crosslinking using TBDSPP result in high sample quality and a large amount of structural information from very complex matrices, with performance comparable to DSPP.
- Modified EasyPep chemistry and peptide-level cleanup can be successfully utilized to increase the number of identified crosslinks and decrease the total amount of sample preparation time

REFERENCES

1. Steigenberger et al. Phox: an IMAC-enrichable Crosslinking Reagent. *ACS Cent. Sci.* **2019**, 5, 1514−1522.
2. Pin Lian Jiang et al. A Membrane-Permeable and Immobilized Metal Affinity Chromatography (IMAC)-Enrichable Cross-Linking Reagent to Advance In Vivo Cross-Linking Mass Spectrometry. *Angew. Chem. Int. Ed.* **2021**, e202113937
3. Bath et al. Protein Aggregation Capture on Microparticles Enables Multipurpose Proteomics Sample Preparation. *Mol. & Cell Proteomics* **2019,** *18*, 1027–1035.

ACKNOWLEDGEMENTS

We would like to thank Richard Scheltema and Barbara Steigenberger from Utrecht University in Utrecht, the Netherlands, for providing crosslinkers and collaborating on sample prep techniques.

TRADEMARKS/LICENSING

PO66163-EN0422S