An optimized enrichment strategy for improved mass spec analysis of chemically crosslinked peptides

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ABSTRACT

Purpose: To optimize the sample preparation and enrichment workflow using an azide-tagged, acid-cleavable disuccinimidyl bis-sulfoxide (aaDSBSO) crosslinker for studying protein-protein interactions.

Methods: Amine-reactive, MS-cleavable crosslinkers including DSSO and aaDSBSO were used to crosslink standard proteins and cell lysates. Crosslinked samples were reduced, alkylated and digested with trypsin/LysC before enrichment and LC-MS analysis. For azido-containing crosslinkers, samples were labeled with different biotinylation probes before reduction, alkylation, and digestion, and enriched using different biotin capture resins. Enriched/fractionated BSA samples were separated using a 15cm Thermo Scientific™ EASY-Spray™ column and Thermo Scientific™ UltiMate™ 3000 UHPLC system in 75min gradient, followed by the detection on the Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ mass spectrometer. Data were analyzed using Thermo Scientific™ Proteome Discoverer™ 2.2 software and XlinkX node.

Results: Our aaDSBSO sample preparation workflow optimized protein crosslinking, biotinylation, enrichment, elution, acid-cleavage and clean up resulting in more crosslinked peptide identifications for BSA and HeLa cell proteins while decreasing the total sample preparation time from 3.5 days to under 2 days.

INTRODUCTION

Chemical cross-linking in combination with mass spectrometry is a powerful method to determine protein structure and protein-protein interactions.[1] This method has been applied to recombinant and native protein complexes and, more recently, to whole cell lysates in efforts to identify protein-protein interactions on a global scale. However, this method suffers from low identification rates without enrichment/fractionation, as typical yields of cross-linked peptides are less than 1% of total identified peptides. In this study, we assessed aaDSBSO, a MS-cleavable crosslinker which can be labeled and enriched using copper-free, click chemistry affinity probes.[2] Specifically, we evaluated different probes, capture resins and elution protocols for crosslinked peptide enrichment to increase cross-linked peptide identification using Orbitrap Fusion Tribrid mass spectrometer.

MATERIALS AND METHODS

Sample Preparation

BS3, DSSO and DSBSO were used to crosslink 2mg/ml BSA in PBS for 1hr at various molar excess of crosslinker to protein. After crosslinking, reactions were quenched with 0.5M ammonium bicarbonate and analyzed by SDS-PAGE or reduced, alkylated and digested with trypsin and lys-C for MS analysis. aaDSBSO samples were biotinylated prior to reduction, alkylation and digestion, with protein-level cleanup steps before and after biotinylation using Thermo Scientific™ Pierce™ Protein 10K MWCO concentrators. Protein and peptide concentrations were determined using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit and the Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay, respectively. aaDSBSO samples were analyzed directly with and without enrichment. Furthermore, DSSO and aaDSBSO BSA samples were spiked into HeLa digests at concentrations ranging from 1-10% prior to enrichment. Enrichment was performed using Pierce™ NeutrAvidin™ Plus Ultralink™ Resin. After elution and acid cleavage, samples were reapplied to NeutraAvidin resin to remove excess biotin prior to LC-MS analysis.

Liquid Chromatography

Samples were separated by RP-HPLC using an UltiMate 3000 system, using an in-line Thermo Scientific™ Acclaim™ PepMap™ 100 C18 LC trap column and a Thermo Scientific™ EASY-Spray™ analytical column, 15 cm x 75 µm over 75 minutes, using a 2-28% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 300nL/min flow rate.

Mass Spectrometry

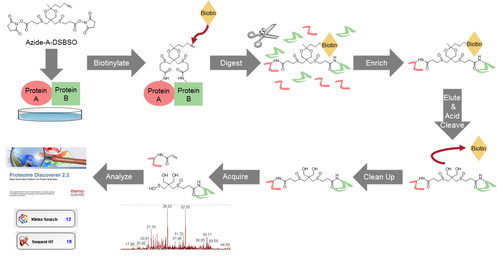
Crosslinked BSA and HeLa peptide samples were analyzed on the Orbitrap Fusion Tribrid mass spectrometer. Acquisition parameters were as follows: OTMS full scan at 120K resolution, 10ppm mass tolerance, AGC target 4.0e5, 50ms injection time, 20s dynamic exclusion. Data dependent MS^2 OT CID was subsequently performed for precursors exhibiting a charge state of 3-6 using 30K resolution, 1.6m/z isolation window, AGC target 5.0e4, 100ms injection time, and 25% CID collision energy. ddMS^2 IT CID with 2.0 m/z isolation, 120ms injection time, AGC target 2.0e4, and 35% CID collision energy.

Data Analysis

Spectral data files were analyzed with Proteome Discoverer 2.2 software using the XlinkX node for crosslinked peptides and SEQUEST®HT search engine for unmodified and monolink peptides. Carbamidomethylation (+57.021 Da) was used as a static modification for cysteine. Crosslinked variable mass modifications for lysine on aaDSBSO samples as follows: aaDSBSO (+308.03883), oxidized (+324.0337), hydrolyzed (+326.04939), alkene (+54.01056), and methionine oxidation (+15.996 Da).

Data were searched against a BSA or human proteome database with a 1% FDR criteria for protein spectral matches. For MS^2-MS^2 methods, a linear–peptide search option (using MS^2 scans for identification and MS^2 scan for detection of crosslinked peptides) was used for XlinkX database searching. XlinkX standard enumeration search was used for data acquired using MS^2 methods.[3]

Figure 1. Schematic of the optimized workflow for aaDSBSO sample crosslinking, biotinylation, digestion, enrichment, elution/acid cleavage, cleanup and LC-MS analysis.



RESULTS

A common method to determine crosslinking efficiency is through decreased mobility by SDS-PAGE. Comparison of BS3, DSSO and aaDSBSO crosslinking of BSA samples shows that aaDSBSO and DSSO have similar crosslinking efficiency, but both are less efficient than BS3 (Figure 2). Overall, a 100-300 fold molar excess of crosslinker to protein was found to be the optimal concentration for BSA. Using these concentrations, we evaluated four different copper-free click biotinylation reagents. As shown in Figure 3, the sDIBO reagent had the highest biotinylation labeling efficiency at lower concentrations than the other reagents tested.

Figure 2. Comparison of BS3, DSSO and aaDSBSO crosslinking efficiency for BSA analyzed by SDS-PAGE and Coomassie stain. BS3 and DSSO samples were prepared at 100X molar excess, and aaDSBSO samples were prepared at concentrations from 10X to 300X molar excess.

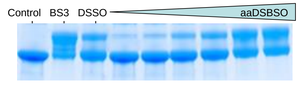


Figure 3. Structures of two copper-free click biotinylation reagents from Life Technologies (A) and two reagents from Click Chemistry Tools (B). (C) Evaluation of copper-free biotinylation reagents for labeling aaDSBSO-crosslinked BSA using streptaviding HRP Western blotting. Probes were incubated for 1hr at room temperature at concentrations from 10µM to 500µM.

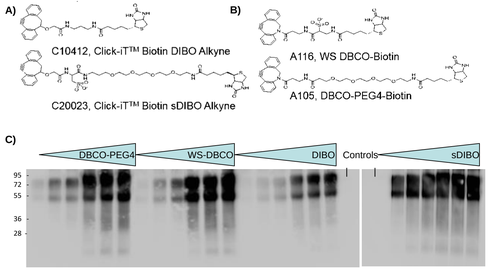
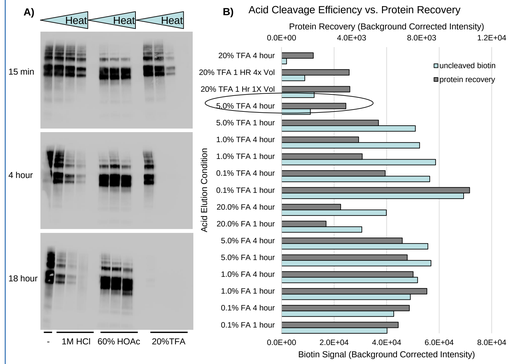


Figure 4. (A) Evaluation of biotinylated, aaDSBSO-crosslinked BSA protein acid cleavage using different acids (HCl, HOAc, FA and TFA), temperatures (22ºC, 37ºC, and 50ºC), and time points analyzed by streptavidin HRP Western blotting. (B) Quantitation of biotin signal normalized to protein recovery analyzed by SDS-PAGE and Coomassie staining.



At the protein level, acid cleavage using 5% TFA for 4 hours at 50˚C was found to be the optimal condition for maximum biotin cleavage with minimal protein loss (Figure 4). However, at the peptide level, heating the samples in high concentrations of TFA longer at elevated temperatures for times greater than 1 hour resulted in a marked decrease in the number of crosslinked peptide spectra and identifications, likely due to peptide hydrolysis. Compared to using the original 20% FA elution conditions, using our optimized elution conditions resulted in increased triggered MS3 scans and 2-3 fold more crosslinked peptide identifications (Figure 5).

Figure 5. Evaluation of biotinylated, aaDSBSO-crosslinked BSA peptide acid cleavage using different acids, temperatures, and time points analyzed by LC-MS/MS. Efficiency was evaluated in terms of number of identifications (A) and intensity of MS3 triggering selectively for aaDSBSO spectra (B).

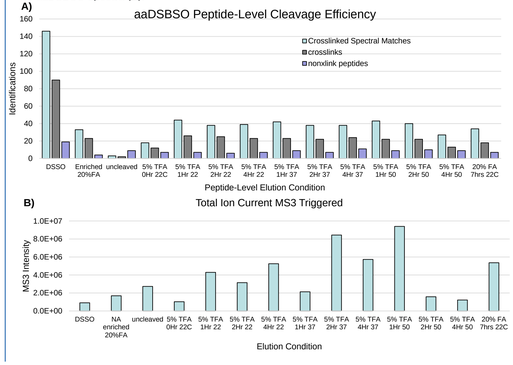
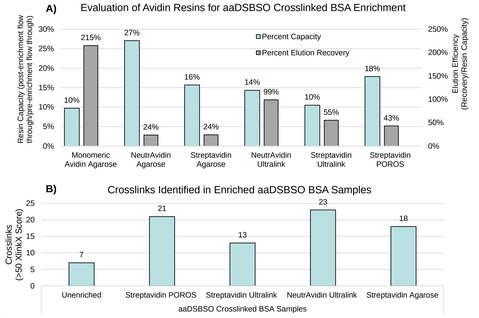
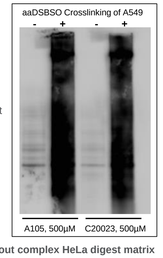


Figure 6. (A) Evaluation of six avidin-based affinity resins for capacity and elution efficiency. (B) Comparison of four avidin-based affinity resins for crosslink peptide identification rates.



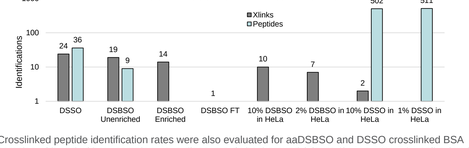
Next, we evaluated six different avidin-based resins for biotinylated, crosslinked peptide enrichment. Monomeric avidin agarose was determined to be unfit for low-acid conditions, demonstrating significant resin destruction (Figure 6, A). Interestingly, NeutrAvidin Agarose and Streptavidin Agarose resins performed well in terms of capacity, but poorly in terms of comparative sample recovery. LC-MS analysis of samples showed the highest number of crosslinks from NeutrAvidin Plus Ultralink and Streptavidin POROS resins (Figure 6, B), both of which performed well in terms of sample capacity and micrograms of peptide recovered from elution.

Figure 7. A549 cell pellets with and without aaDSBSO crosslinking, lysis and subsequent biotinylation using two selected reagents to assess nonspecific biotinylation by streptavidin HRP Western Blotting



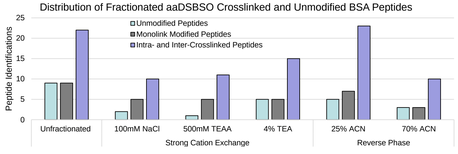
In order to understand membrane permeability of aaDSBSO and relative rates of non-specific biotinylation activity in complex matrices, we evaluated in-vitro crosslinking of cells and subsequent biotinylation using two biotinylation probes with similar rates of biotinylation efficiency. A549 cells were crosslinked with 200x molar excess of aaDSBSO or left at 22C without crosslinking for 1 hour. Crosslinked and non-crosslinked samples were lysed in 1.0% SDS and 50mM TEAB, and both crosslinked and non-crosslinked lysates were biotinylated with either biotinylation reagent A105 or C20023. Results indicate that aaDSBSO is cell-permeable, and biotinylation with C20023 results in 42% less non-specific biotinylating of A549 cells.

Figure 8. aaDSBSO and DSSO crosslinked BSA with and without complex HeLa digest matrix and subsequent enrichment



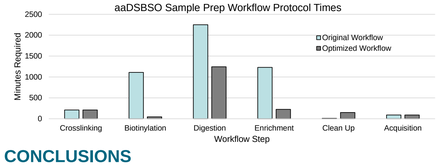
Crosslinked peptide identification rates were also evaluated for aaDSBSO and DSSO crosslinked BSA samples with and without complex HeLa digest matrix background (Figure 8). At a 10% BSA spike (w:w), DSSO samples showed a significant loss in the number of crosslinked peptide identifications. In contrast, the 10% BSA spike aaDSBSO samples demonstrated almost 50% recovery of crosslinked peptide identifications compared to crosslinked BSA only control samples without HeLa matrix. At a 2% spike of aaDSBSO crosslinked BSA, 37% of crosslinked identifications are still observed. Only a single BSA crosslinked peptide was identified in the flow through with no unmodified peptides after enrichment, demonstrating high specificity.

Figure 9. Comparison of Reverse-Phase and SCX fractionation workflows to enhance crosslinked peptide identifications with aaDSBSO crosslinked BSA.



Compared to unfractionated aaDSBSO crosslinked BSA samples, SCX fractionation with 100mM NaCl and 4% TEA resulted in 14% more crosslinked peptide identifications and 33% fewer unmodified identifications, while Reverse-Phase fractionation resulted in 50% more crosslinked peptide identifications and 11% fewer unmodified identifications. (Figure 9). Our optimized workflow for aaDSBSO significantly reduces the sample preparation time from 3.5 days to under 2 days, a 60% improvement in total protocol time (Figure 10).

Figure 10. Comparison of optimized aaDSBSO sample preparation workflow to original protocol.



CONCLUSIONS

- The enrichable, crosslinker aaDSBSO, crosslinks BSA with similar efficiency compared to DSSO; however, both have ~30% lower crosslinking efficiency than BS3.
- Four different copper-free click biotinylation reagents were evaluated for aaDSBSO labeling efficiency, reaction time, and non-specific biotinylation, with the sDIBO PEG having the best specific reactivity.
- Six different avidin-based resins and multiple elution conditions were compared for biotin enrichment, elution and acid cleavage. Optimized conditions resulted in a -shorter protocol time (<2 days vs 3.5 days), higher peptide capture and recovery, more efficient cleaved biotin removal, higher crosslinked peptide identifications, and a lower number of non-specific, unmodified peptides.
- Enrichment of aaDSBSO crosslinked BSA samples spiked into HeLa digest resulted in 5-fold higher crosslinked peptides identified when compared to DSSO samples in the same matrix without enrichment.

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