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Optimized Fragmentation Improves the Identification of Peptides Cross-Linked by MS-Cleavable Reagents

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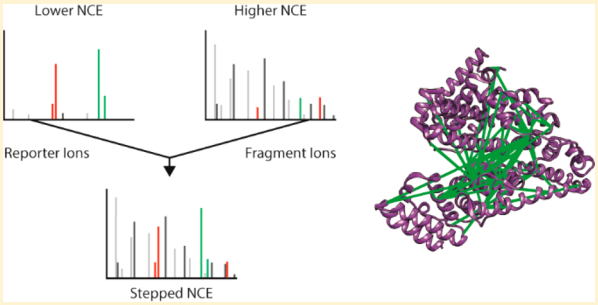
Supporting Information

ABSTRACT: Cross-linking mass spectrometry is becoming increasingly popular, and current advances are widening the applicability of the technique so that it can be utilized by nonspecialist laboratories. Specifically, the use of novel mass-spectrometry-cleavable (MS-cleavable) reagents dramatically reduces the complexity of the data by providing (i) characteristic reporter ions and (ii) the mass of the individual peptides rather than that of the cross-linked moiety. However, optimum acquisition strategies to obtain the best-quality data for such cross-linkers with higher energy C-trap dissociation (HCD) alone are yet to be achieved. Therefore, we have carefully investigated and optimized MS parameters to facilitate the identification of disuccinimidyl-sulfoxide-based cross-links on HCD-equipped mass spectrometers. From the comparison of nine different fragmentation energies, we chose several stepped-HCD fragmentation methods that were evaluated on a variety of cross-linked proteins. The optimal stepped-HCD method was then directly compared with previously described methods using an Orbitrap Fusion Lumos Tribrid instrument using a high-complexity sample. The final results indicate that our stepped-HCD method is able to identify more cross-links than other methods, mitigating the need for multistage MS-enabled (MS^n) instrumentation and alternative dissociation techniques. Data are available via ProteomeXchange with identifier PXD011861.



KEYWORDS: *cross-linking mass spectrometry, XLMS, cleavable cross-linker, stepped HCD, DSSO*

INTRODUCTION

Cross-linking mass spectrometry (XLMS) is a rapidly growing field of research at the interface of proteomics and structural biology.[1−5] Typically, *N*-hydroxysuccinimide (NHS)-based functionalities that link primary amines (lysine, protein N-terminus) and hydroxyl groups (serine, threonine, and tyrosine) are used as reactive groups to form covalent bonds between residues that are in close spatial proximity. After reacting proteins, protein complexes, or even whole cells with one of these reagents, the sample is digested enzymatically. This results in a complex mixture containing linear peptides, mono- or dead-end links (peptides that reacted with one end of the cross-linker while the other reactive group is hydrolyzed), intrapeptide cross-links (peptides containing two linkable amino acids without an enzymatic cleavage site in-between), and interpeptide cross-links (cross-links that link two separate peptides). This mixture is then analyzed via high-performance liquid chromatography (LC)−tandem mass spectrometry (MS/MS or MS^2) to identify the cross-linked species. Because cross-linked peptides are formed substoichiometrically, mass spectrometers offering high sensitivity and high scan rates are required for a comprehensive analysis.

As well as their detectability, the estimation of the false discovery rate (FDR) for cross-linked peptides is also more challenging compared with linear peptides.[6,7] Over the past decade, several MS-cleavable cross-linkers have been developed that facilitate data analysis and diminish the possibility of false-positives.[8−11] The two most commonly used and commercially available MS-cleavable cross-linkers, disuccinimidyl sulfoxide (DSSO, Figure 1) and disuccinimidyl dibutyric urea (DSBU or BuUrBu, Figure 1) have been extensively investigated, and because of their beneficial features, they simplify the analysis of cross-links. Both contain chemical groups that cleave upon collisional activation. DSBU is the diamide of carbonic acid and aminobutanoic acid. These amide bonds have a stability comparable to that of the amide bonds in the peptide backbone. Therefore, higher energy C-trap dissociation (HCD), a beam-type collision-induced dissociation method, is the fragmentation method of choice and is frequently applied in the measurement of DSBU cross-linked peptides.[12,13] In contrast, the C−S bonds adjacent to the sulfoxide group of DSSO are weaker than the peptide

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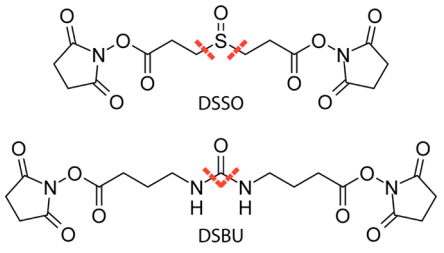

Figure 1. Illustration of disuccinimidyl sulfoxide (DSSO) and disuccinimidyl dibutyric urea (DSBU). Red dotted lines indicate collision-induced-dissociation-associated cleavage sites.

backbone and can be selectively cleaved upon collision-induced dissociation (CID).

To obtain satisfying sequence coverage, required for unambiguous cross-link identification, a second MS/MS scan with a complementary fragmentation method such as electron-transfer dissociation (ETD) or separate sequencing of the arising reporter doublets in third-stage mass spectrometry (MS^3) is often acquired.[14,15] The disadvantages of sequential MS/MS scans or multistage MS (MS^n) methods are reduced scan rate and lower sensitivity. Moreover, advanced instruments like Orbitrap Fusion or Orbitrap Fusion Lumos are required for optimal performance. Previous experiments have already described HCD to yield the highest number of identified cross-links for noncleavable cross-linking reagents.[16,17] These studies impressively demonstrated that optimal fragmentation is crucial for cross-link analysis and allows the identification of up to four times more cross-linked peptide pairs. Moreover, they highlight HCD to be the fragmentation strategy of choice for most cross-linked species.

A recent publication by Smith et al.[18] gives a detailed comparison on different fragmentation methods and data analysis tools for peptides cross-linked with both DSSO and DSBU. This comparison also includes two different fragmentation approaches, namely, stepped-HCD and sequential CID-ETD fragmentation; however, this study did not investigate the collision energy dependency on the fragmentation of DSSO cross-linked peptides, and optimization of the stepped-HCD acquisition strategy was not performed. Moreover, in this study, two particular proteins have been used, which yielded a limited number of cross-links.

In the study presented herein, we have elucidated the influence of different normalized collision energies (NCEs) on the HCD fragmentation behavior of DSSO cross-linked peptides. Therefore, the targeted analysis of cross-linked peptides derived from five different proteins, rabbit aldolase, bovine serum albumin (BSA), equine myoglobin, *S. pyogenes* Cas9, and human transferrin, was carried out. Cross-links for targeted analysis were identified by using the previously published sequential CID-ETD acquisition method and XLinkX 2.2 for the database search.[15] On the basis of the fragmentation behavior, search engine scores, and identified cross-linked species, different stepped collision energies were proposed and compared to each other. Finally, the optimal performing stepped collision energy was compared to published acquisition strategies for DSSO[14,15] using two commercially available systems, BSA, which served as a model system for a single protein, and the 70S *E. coli* ribosome, which served as a model system for a more complex sample.

MATERIALS AND METHODS

Chemicals and Reagents

Aldolase (rabbit), alcohol dehydrogenase (ADH, yeast), BSA, conalbumin (chicken), myoglobin (equine), ovalbumin (chicken), and transferrin (human) were purchased from Sigma-Aldrich (St. Louis, MO), *E. coli* 70S ribosome from New England Biolabs (Ipswich, MA), and DSSO from Thermo Fisher Scientific (Rockford, IL). Cas9 (*S. pyogenes*) with a fused Halo tag was expressed and purified as described by Deng et al.[19]

Cross-Linking and Digestion

Prior to cross-linking, Cas9 was buffer-exchanged to XL buffer (50 mM HEPES, 50 mM KCl, pH 7.5) using Micro Bio-Spin 6 columns (Bio-Rad, Hertfordshire, U.K.). The other proteins were dissolved in XL buffer. (The ribosome sample contained an additional 10 mM $MgAc_2$.) All proteins were cross-linked separately at a concentration of 1 μg/μL and a final DSSO concentration of 500 μM. The reaction was carried out for 1 h at room temperature before it was quenched with 50 mM Tris-HCl, pH 7.5. Samples were reduced with dithiothreitol (DTT, 10 mM, 30 min, 60 °C) and alkylated with iodoacetamide (IAA, 15 mM, 30 min at room temperature in the dark). Alkylation was stopped by the addition of 5 mM DTT, and proteins were digested with trypsin overnight (protein/enzyme 30:1, 37 °C). Digestion was stopped by the addition of trifluoracetic acid to a final concentration of 1% (v/v, pH <2), and samples were stored at −80 °C. The sample for stepped-HCD comparison was obtained by mixing all digests in an equimolar ratio.

For the sequential digest, tryptic peptides were desalted using self-made C-18 Stage Tips,[20] dissolved in XL buffer, and digested with *S. aureus* Protease V8 (GluC) for 4 h (protein/enzyme 30:1, 37 °C).

Size-Exclusion Chromatography Enrichment

The ribosome sample was enriched for cross-links (XLs) prior to LC−MS/MS analysis using size-exclusion chromatography (SEC). Twenty-five μg of the digest was separated on a TSKgel SuperSW2000 column (300 mm × 4.5 mm × 4 μm, Tosoh Bioscience) (Figure S8). The three high mass fractions were subsequently measured via LC−MS/MS.

Reversed-Phase High-Performance Liquid Chromatography

Digested peptides were separated using a Dionex UltiMate 3000 high-performance liquid chromatography (HPLC) RSLCnano System prior to MS analysis. The HPLC was interfaced with the mass spectrometer via a Nanospray Flex ion source. For sample concentrating, washing, and desalting, the peptides were trapped on an Acclaim PepMap C-18 precolumn (0.3 × 5 mm, Thermo Fisher Scientific) using a flow rate of 25 μL/min and 100% buffer A (99.9% H_2O, 0.1% TFA). The separation was performed on an Acclaim PepMap C-18 column (50 cm × 75 μm, 2 μm particles, 100 Å pore size, Thermo Fisher Scientific) applying a flow rate of 230 nL/min. For separation, a solvent gradient ranging from 2 to 35% buffer B (80% ACN, 19.92% H_2O, 0.08% TFA) was applied. The applied gradient varied from 60 to 180 min, depending on the sample complexity.

Mass Spectrometry

All measurements involving DSSO and DSBU optimization and comparison were performed on an Orbitrap Fusion Lumos

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Tribrid (Thermo Fisher Scientific) mass spectrometer. The identification of the proteins contained in the ribosome sample as well as the identification of cross-links from BSA (DSBU), ADH (DSSO), conalbumin (DSSO), and ovalbumin (DSSO) were performed on QExactive Orbitrap (Thermo Fisher Scientific) mass spectrometers. For detailed procedures, see the Supporting Information.

Data-Dependent Acquisition Methods

For DSSO-XL identification, digested proteins were analyzed using the CID-ETD acquisition method described by Liu et al.[14] Survey scans were recorded at 60 000 resolution and a scan range from 375 to 1500 m/z (AGC 4e5, max injection time 50 ms). MS/MS scans were recorded at 30 000 resolution (AGC 5e4, max injection time 100 ms for CID and 120 ms ETD, isolation width 1.6 m/z). Singly and doubly charged ions were excluded from fragmentation because cross-linked peptides tend to occur at a charge state of 3+ or above.[21] The CID fragmentation energy was set to 25% NCE, and for ETD, calibrated charge-dependent ETD parameters were used. In CID-ETD acquisition, two subsequent fragmentation events using the complementary fragmentation strategies were triggered. All precursors that have been selected for fragmentation were excluded from fragmentation for 30 s.

For the MS^n acquisition strategy (called MS^2-MS^3 from now on), the same settings as those described above were used, but only precursors with charge states 4−8+ were selected for MS/MS. The two most abundant reporter doublets from MS/MS scans (charge states 2−6, Δ-mass 31.9721 Da, ±30 ppm) were selected for MS^n. MS^3 scans were recorded in the ion trap operated in rapid mode with a maximum fill time of 150 ms (isolation width 2.0 m/z). Fragmentation was carried out using HCD with 30% NCE.

For stepped-HCD the settings described above were used with one adaptation. Ions for MS/MS were collected for a maximum of 150 ms. Selected precursors were fragmented by applying a collision energy of 27 ± 6% NCE.

NCE Optimization

An inclusion list was generated that included all cross-links identified with CID-ETD using the Proteome Discoverer 2.2 output. Survey scans were recorded at a resolution of 120 000 ranging from 400 to 1600 m/z (AGC 2e5, 50 ms max. injection time). Only precursors from the inclusion list (10 min retention time window, matching charge, and m/z [±10 ppm]) were selected for fragmentation. MS/MS spectra were recorded at 30 000 resolution (AGC 1e5, max. injection time 150 ms, isolation width 1.4 m/z). Each selected precursor was fragmented consecutively with nine different NCEs and subsequently excluded from fragmentation for 30 s. The chronological order of fragmentation energies was randomly shuffled between the three injection replicates (Figure 2).

To identify the optimal stepped-HCD method for the analysis of DSSO cross-linked peptides, a mixture of eight proteins was analyzed in triplicate using three different stepped NCEs. To allow an unbiased comparison and to compensate for chromatographic variations, only precursors from an inclusion list (for the combined inclusion list from separate proteins, see the Effect of NCE on XL Identification section, and for the three new proteins, see the Supporting Information) were selected for fragmentation. For each precursor, three subsequent fragmentation events using different stepped-HCD methods were triggered. Measurements were carried out in triplicate with shuffled fragmentation order. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[22] partner repository with the data set identifier PXD011861.

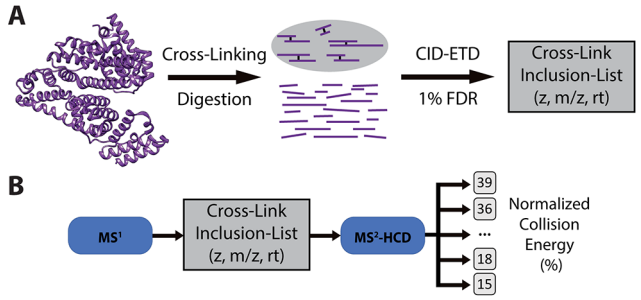

Figure 2. (A) Illustration of the used workflow, adopted from Kolbowski et al.[16] Proteins were cross-linked separately and analyzed one by one on an Orbitrap Fusion Lumos with the already published sequential CID-ETD method. (B) An inclusion list was generated and used for a subsequent targeted analysis of all identified cross-linked peptides using HCD with different normalized collision energies ranging from 15 to 39%.

Data Analysis

Thermo .raw files were imported into Proteome Discoverer 2.2 and analyzed with XLinkX (version 2.2 or 2.3) using the following settings: Cross-Linker: DSSO (+158.00376 Da, reactivity toward lysine and protein N-terminus for initial identification and NCE optimization; for method comparison, serine, threonine, and tyrosine were additionally included); cross-linker fragments: alkene (+54.01056 Da), unsaturated thiol (+85.98264 Da), sulfenic acid (+103.9932 Da); cross-link doublets: alkene/unsaturated thiol (Δ-mass 31.96704 Da) or alkene/sulfenic acid (Δ-mass 49.98264 Da); full-scan accuracy: 10 ppm; MS^2 accuracy: 20 ppm; MS^3 accuracy: 0.5 Da; used enzyme: trypsin; max. missed cleavages: 4; minimum peptide length: 5; max. modifications: 4; peptide mass: 300−7000 Da; static modifications: carbamidomethylation (cysteine, +57.021 Da); dynamic modifications: oxidation (methionine, +15.995 Da). For the database search, the FDR was set to 1%. To reduce the number of false-positives, cross-links identified with XLinkX were filtered for an identification score ≥20, as suggested by Thermo Fisher and additionally for an identification delta score (Δ-score) ≥20. CID-ETD and MS^2−MS^3 runs were analyzed with the MS2_MS2 or MS2_MS3 workflow provided in Proteome Discoverer 2.2. Detailed analysis parameters using MeroX are described in the Supporting Information.

The FASTA files for the database search contained the used model proteins and all identified proteins contained in the ribosome sample, respectively.

RESULTS

Effect of NCE on XL Identification

We first sought to investigate the identification rate of DSSO cross-linked peptides with respect to the NCE employed during HCD activation. Therefore, we adopted the workflow described by Kolbowski et al.[16] (Figure 2). To achieve optimal reproducibility, an inclusion list of previously identified cross-link precursors was generated for each protein used in the study (Aldolase, BSA, Cas9, Myoglobin, Transferrin). The detection of an XL precursor triggered nine consecutive

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fragmentation events utilizing different NCEs, ranging from 15 to 39% (Figure 2). Samples were measured in triplicate, with randomly shuffled collision energy order. The recorded data set was analyzed using XLinkX 2.2,[15] and all runs were searched against a database containing the five investigated proteins.

On average, NCEs of 21 and 24% could identify the highest number of cross-linked sites (e.g., unique linked amino acids; 293/289) when applying a 1% FDR (Figure 3). The employment of higher NCEs (>27%) leads to a significantly lower cross-link identification, with 39% NCE identifying over 70% fewer than the two best collision energies (21 and 24%). The score provided by XLinkX reaches its maximum between 27 and 30% NCE (Figure 3). In addition to XLinkX, the data set was also analyzed with MeroX 1.6.6.[13] This algorithm gave similar results, with a slightly shifted ID maximum at 24% NCE (Figure S1).

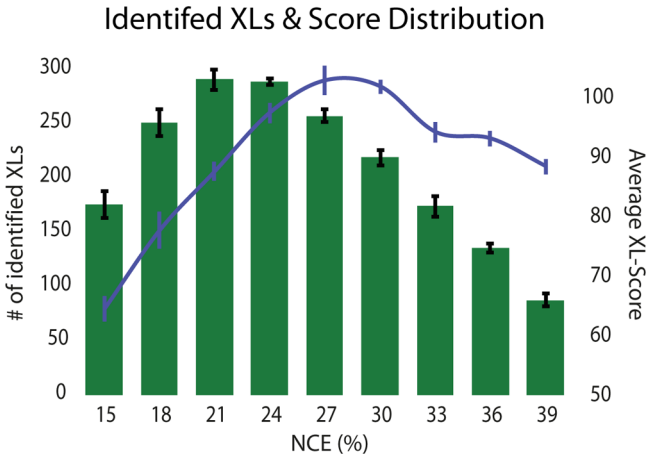

Figure 3. Number of identified cross-links (green) and their average score (blue) according to different the normalized collision energies (n = 3, error bars represent the 0.95 confidence interval (CI)).

Because scoring is highly dependent on the scoring function implemented in the search engine, we aimed for a more independent measure to compare the different fragmentation energies. Hence, we compared the sequence coverage obtained with different NCEs. The data were analyzed with XLinkX 2.3 in PD 2.3 (beta), and the reported sequence coverage for the identified XLs was used for comparing different NCEs. An overall comparison of the sequence coverage already shows that higher NCEs provide higher sequence coverage (Figure 4A).

To investigate the relationship between the charge state and m/z range of a given peptide with its fragmentation behavior, the cross-link identifying spectra (CSMs) were sorted into separate groups according to charge and m/z for further comparison. Only the highest-scoring CSM for each cross-linked m/z species (e.g., the same cross-linked peptide at different charge states) identified in the corresponding replicate was used for analysis. The sequence coverage of different groups revealed a strong dependency of fragmentation behavior on charge density, as has been previously described.[16,21] Lower charge states (3/4+) and m/z (<750 for 3+ and <700 for 4+) result in >70% sequence coverage, already at low collision energies starting from 18% NCE (Figure S2). On the contrary, an m/z above 700 leads to poor precursor fragmentation and therefore to a lower identification rate for low NCEs, as can be seen in Figure S2 for NCE 15 and 18%. The average sequence coverage reaches a plateau between NCE 27 and 33% and does not significantly increase with higher collision energies. When comparing the fragmentation of the two linked peptides, a similar

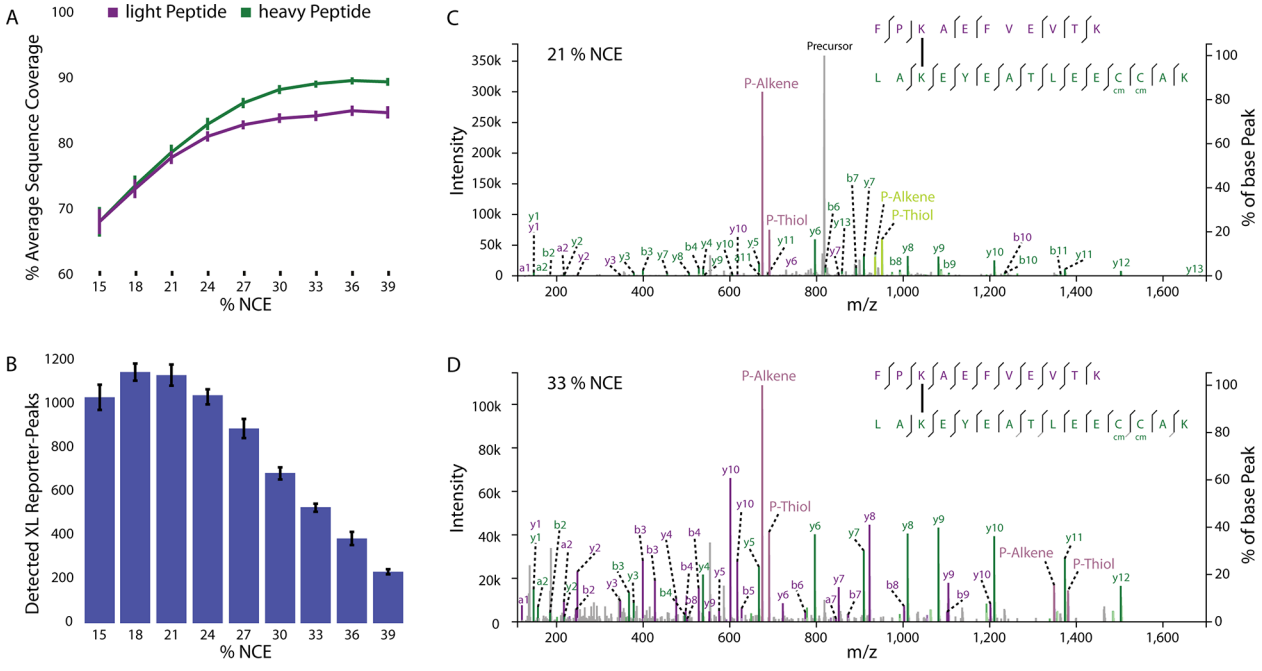

Figure 4. (A) Average sequence coverage of the identified cross-links at different normalized collision energies. (B) Spectra that have been identified to contain at least two different cross-link reporter doublets as a function of the used normalized collision energy. (C,D) Two MS/MS spectra triggered from the same precursor using a rather low ((C) 21% NCE) and a more elevated ((D) 33% NCE) normalized collision energy (n = 3, error bars represent the 0.95 CI). Spectra were annotated with the help of xiSPEC (https://spectrumviewer.org/).[23]

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fragmentation behavior was observed. Nevertheless, the heavier/larger peptide showed a slightly better sequence coverage for all m/z ranges and most fragmentation energies, as observed for noncleavable cross-linking reagents.[21]

The average sequence coverage as well as the scoring of all three used search algorithms indicate that most unambiguous cross-link identifications can be obtained at NCEs above 27%, whereas the most CSMs and cross-links could be identified between 21 and 24% NCE.

Manual inspection of MS/MS spectra revealed that at least one of the two reporter ion doublets is absent at higher NCEs, whereas the relative intensity of the fragment ions increases with rising NCE (Figure 4C,D). A more comprehensive investigation into the presence of reporter ion doublets confirmed these observations. Whereas NCEs from 15 to 24% on average yielded more than 1000 MS/MS spectra containing at least one reporter doublet for each peptide, 39% NCE could generate only 237 (Figure 4B). These doublets are essential for identification by the algorithm employed, which is why lower NCEs are able to identify more cross-linked peptides than higher NCEs. Unlike the number of identifications, the conversion rate of MS/MS scans containing reporter ion doublets to CSMs increases from roughly one-third at 15% NCE to 86% for NCEs >30%.

In the case of DSSO, it has been previously assumed that CID is the most suitable fragmentation technique for reporter doublet formation.[9,15] Therefore, HCD was compared to CID fragmentation using BSA (n = 3). The previously generated inclusion list was used for this comparison, with CID (30% NCE) included as 10th fragmentation event. The results show that HCD fragmentation using lower NCEs (15−24%) results in the same number of reporter doublets as CID fragmentation (Figure S3). Nevertheless, it must be noted that there might be DSSO cross-linked peptides that do not form reporter doublets with either CID or HCD.

Finally, we investigated the fragmentation efficiencies of different NCEs. With ≥27% NCE, almost no precursor ion remained after fragmentation (<1%). In contrast, the three lowest NCEs tested (15−21%) resulted in MS/MS spectra where on average the precursor ion corresponds to 55, 38, or 19% of all detected ions, respectively (Figure S4).

Seemingly, there is not one specific NCE that is suitable for HCD fragmentation of DSSO cross-linked peptides. However, after merging all of the different results, we concluded that fragmentation energies below 21% and above 33% NCE can be neglected because they do not offer unique properties or benefits but rather come with several disadvantages. Most likely, a stepped collision energy, which combines reporter ion formation (NCE 21/24%) as well as high sequence coverage (>27% NCE) in one fragmentation event, will yield the optimal result, as previously described for the identification of post-translationally modified peptides.[13,24,25]

The collision-energy-dependent fragmentation of peptides cross-linked with the second commercially available cleavable cross-linking reagent DSBU was also investigated. (For the detailed procedure, see the Supporting Information.) On the basis of cross-links derived from BSA (n = 3), the different NCEs were compared for the number of identified cross-linked sites, their average scoring, and the number of spectra containing at least one reporter doublet for both peptides. The results show that all three parameters are significantly less influenced by the fragmentation energy compared with cross-links formed with DSSO (Figure S5). Whereas DSSO cross-linked peptides are less likely to form reporter doublets when fragmented with NCEs >27% (Figure 4B), this effect is less critical for DSBU cross-linked peptides. However, too-low fragmentation energies (<21% NCE) result in an up to 10-fold decreased number of identifications. These results show that the existing fragmentation strategies for DSBU applying NCEs between 25 and 35% already cover the optimal NCE range. Therefore, we did not further investigate the fragmentation behavior of DSBU.

Stepped Collision Energy Comparison

On the basis of our observations, we tested three stepped collision energies that span the NCEs optimal for both doublet formation and sequence coverage (24 ± 3, 27 ± 3, and 27 ± 6% NCE). For comparison, a mixture of all investigated proteins was used. To allow an unbiased comparison, only precursors from an inclusion list (for a combined inclusion list from separate proteins, see the Effect of NCE on XL Identification section) were selected for fragmentation. For each precursor, three subsequent fragmentation events were triggered.

The method using 27 ± 6% NCE identified the most CSMs in all three replicates. The average number of CSMs increased by >11% from 24 ± 3% NCE to 27 ± 6% NCE, with 27 ± 3% NCE identifying 6% fewer (Figure 5). Also, when using MeroX for data analysis, the highest number of CSMs was identified using 27 ± 6% NCE (Figure S3). To summarize, using an NCE of 27 ± 6% yields the highest number of identifications and therefore is the fragmentation energy of choice for the MS^2-based identification of DSSO cross-linked peptides.

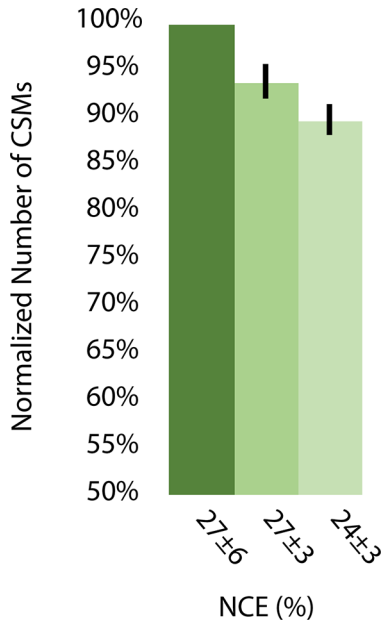

Figure 5. Comparison of the three most promising normalized collision energies for stepped HCD (n = 3, error bars represent the 0.95 CI).

Comparison with Other Methods

Stepped-HCD was compared to the previously described methods using BSA and the 2.5 MDa 70S *E. coli* ribosome that represents an ideal model for large protein complexes. Because manual inspection of CSMs revealed that those with a low Δ score (<40) mostly show poor fragment ion series, the results

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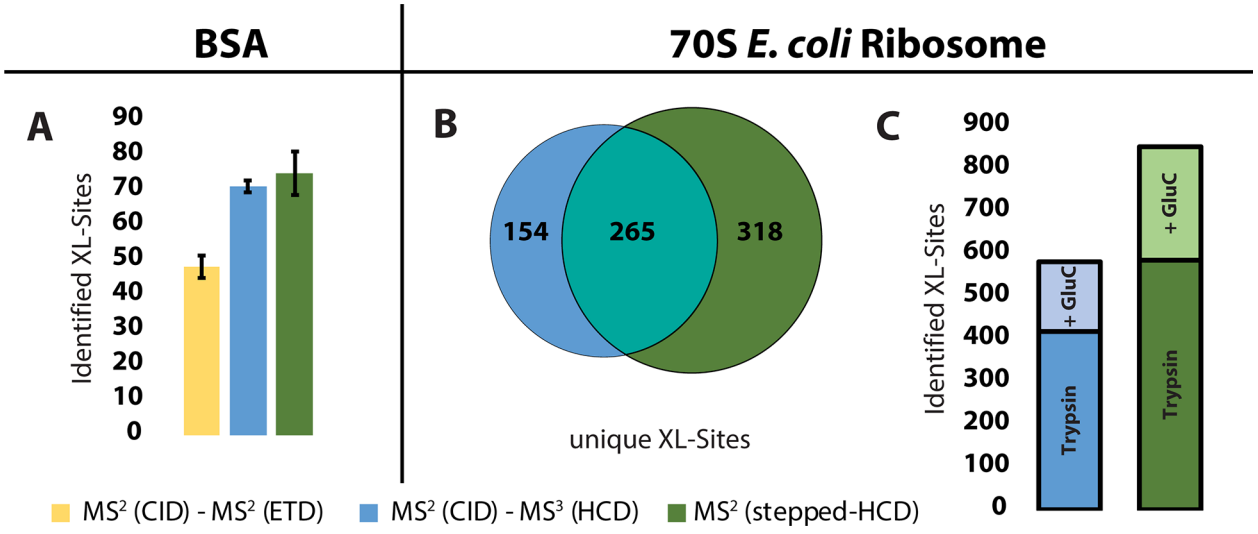

Figure 6. (A) Comparison of the stepped-HCD approach with previously published methods on BSA (n = 3, error bars represent the 0.95 CI). (B) Venn diagram showing the overlap of unique XL sites identified in the tryptic digest using stepped-HCD and MS²−MS³. (C) Total number of identified XL sites identified with MS²−MS³ and stepped HCD, respectively. Additional unique XL sites obtained from the sequential digest using trypsin and GluC are indicated in light blue and light green, respectively.

were filtered for Δ score ≥40. The cross-linked BSA digest was measured in triplicate, and for the ribosome, SEC fractions 2 and 3 were measured in triplicate.

In the case of BSA, the newly developed stepped-HCD method identified an average of 75 unique cross-linked sites, statistically not significantly more than MS²−MS³, which identified an average of 71. These results are in the same magnitude as suggested by a community-wide XLMS study.[26] However, both outperformed the approach employing two complementary fragmentation types CID-ETD by 56 and 48%, respectively (Figure 6A). Therefore, this approach was not included for the comparison of the more complex sample.

To further evaluate the performance of the stepped-HCD, it was compared to the MS²−MS³ method on cross-links derived from the 2.5 MDa 70S *E. coli* ribosome. The digest was enriched for cross-linked peptides using SEC. 583 unique cross-linked sites were identified using the stepped-HCD method applying a 1% FDR. Meanwhile, MS²−MS³ identified 28% fewer (419) sites when using the same parameters (Figure 6B). However, only 36% (265) of the cross-linked sites are shared between both methods, whereas 318 and 154 are uniquely identified by stepped-HCD and MS²−MS², respectively. This indicates a certain degree of complementarity of the two strategies, as was previously reported for other CID-cleavable cross-linking reagents.[27] Hence a combination of both strategies is likely to lead to a more comprehensive cross-linking result. The advantage of stepped-HCD over the MS²−MS³ acquisition is partially due to the inclusion of triply charged precursors; 44 unique cross-linked sites were exclusively identified using stepped-HCD and were exclusively at charge state 3+. When filtering the results for triply charged precursors, stepped-HCD identified 522 unique sites and still outperformed MS²−MS³ by >20%, although time was wasted acquiring 3+ precursors. Additionally, replicate analysis of SEC-fractions 2 and 3 confirms the superiority of stepped-HCD (Figure S7).

Recent literature highlights the benefits of sequential digestion using different proteases for more comprehensive cross-link identification through enhanced protein sequence coverage.[28] Therefore, the DSSO cross-linked ribosome was additionally digested sequentially using trypsin and GluC. A direct comparison of the two acquisition strategies pointed out the versatility of stepped-HCD (Figure 6C). Whereas the MS²−MS³ method identified 195 unique cross-linked sites, stepped-HCD identified more than twice as many (431 unique sites), again at 1% FDR. This advantage is likely due to the shorter peptides produced by the sequential digest. Hence, cross-links tend to occur at lower charge states (predominantly 3+) that have not been considered for MS²−MS³. In summary, stepped-HCD allowed the identification of 849 unique XL sites, 320 more than the MS²−MS³ approach.

CONCLUSIONS

MS analysis of peptides cross-linked with the cleavable cross-linker DSSO comes with several challenges. First, C−S bonds adjacent to the sulfoxide group are more labile than the peptide bonds, so different fragmentation energies are required for simultaneous cross-linker and peptide cleavage. Second, high fragmentation energies result in the loss of the cross-link reporter doublet ions. Therefore, three stepped collision energies that combine higher and lower fragmentation energies were tested. The best-performing acquisition strategy using 27 ± 6% NCE was subsequently compared to previously described acquisition strategies. This approach was shown to be able to identify more cross-linked sites than other acquisition strategies. In the case of BSA, stepped-HCD performed equally as well as the previously published MS²−MS² method while being the simpler strategy. For the 70S *E. coli* ribosome, a large multisubunit riboprotein, stepped-HCD identified 584 cross-linked sites using a tryptic digest and thereby outperformed the MS²−MS³ acquisition method that identified 417 cross-linked sites only. In addition, it proved to be compatible with sequential digests using multiple proteases, allowing a more comprehensive cross-link analysis. Altogether, our novel fragmentation strategy identified almost 850 unique cross-linked sites, 45% more than the MS²−MS³ method.

Our approach represents a powerful alternative to previously described analysis strategies for DSSO cross-linked peptides. It

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allows their analysis on mass spectrometers equipped with an HCD-cell without the need for ETD or MS^n. Thereby, it will help to make XLMS available to a broader audience. Additionally, this new approach can, in principle, be applied to every other sulfoxide-containing cross-linking reagent.[29−31]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00947.

Supplementary Methods: Analysis parameters used for MeroX, MS acquisition and analysis parameters used for DSBU samples, and MS acquisition and analysis parameters used for shotgun analysis of the ribosome sample. Supporting Figure 1: Mean number of identified CSMs and unique XLs identified at the respective NCE and their average scoring. Supporting Figure 2: Average sequence coverage obtained at different NCEs. Supporting Figure 3: Number of reporter-doublet-containing spectra obtained with CID (30% NCE) and HCD fragmentation applying NCEs ranging from 15 to 39%. Supporting Figure 4: Average fragmentation efficiency obtained at different NCEs. Supporting Figure 5: Investigation into the fragmentation-energy-dependent identification of DSBU cross-linked peptides. Supporting Figure 6: Comparison of the three most promising stepped collision energies. Supporting Figure 7: Identified cross-linked sites identified in SEC fractions 2 and 3 obtained using the different enzyme (combinations) and fragmentation strategies. Supporting Figure 8: UV chromatogram of the SEC fractionation of the ribosomal sample (PDF)

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD011861.

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■ REFERENCES

(1) Rappsilber, J. The Beginning of a Beautiful Friendship: Cross-Linking/mass Spectrometry and Modelling of Proteins and Multi-Protein Complexes. *J. Struct. Biol.* **2011**, *173* (3), 530−540.

(2) Yu, C.; Huang, L. Cross-Linking Mass Spectrometry: An Emerging Technology for Interactomics and Structural Biology. *Anal. Chem.* **2018**, *90* (1), 144−165.

(3) Sinz, A.; Arlt, C.; Chorev, D.; Sharon, M. Chemical Cross-Linking and Native Mass Spectrometry: A Fruitful Combination for Structural Biology. *Protein Sci.* **2015**, *24* (8), 1193−1209.

(4) Leitner, A.; Faini, M.; Stengel, F.; Aebersold, R. Crosslinking and Mass Spectrometry: An Integrated Technology to Understand the Structure and Function of Molecular Machines. *Trends Biochem. Sci.* **2016**, *41* (1), 20−32.

(5) Holding, A. N. XL-MS: Protein Cross-Linking Coupled with Mass Spectrometry. *Methods* **2015**, *89*, 54−63.

(6) Fischer, L.; Rappsilber, J. Quirks of Error Estimation in Cross-Linking/Mass Spectrometry. *Anal. Chem.* **2017**, *89* (7), 3829−3833.

(7) Iacobucci, C.; Sinz, A. To Be or Not to Be? Five Guidelines to Avoid Misassignments in Cross-Linking/Mass Spectrometry. *Anal. Chem.* **2017**, *89* (15), 7832−7835.

(8) Petrotchenko, E. V.; Serpa, J. J.; Borchers, C. H. An Isotopically Coded CID-Cleavable Biotinylated Cross-Linker for Structural Proteomics. *Mol. Cell. Proteomics* **2011**, *10* (2), M110.001420.

(9) Kandur, W. V.; Kao, A.; Vellucci, D.; Huang, L.; Rychnovsky, S. D. Design of CID-Cleavable Protein Cross-Linkers: Identical Mass Modifications for Simpler Sequence Analysis. *Org. Biomol. Chem.* **2015**, *13* (38), 9793−9807.

(10) Tang, X.; Bruce, J. E. A New Cross-Linking Strategy: Protein Interaction Reporter (PIR) Technology for Protein-Protein Interaction Studies. *Mol. BioSyst.* **2010**, *6* (6), 939−947.

(11) Müller, M. Q.; Dreiocker, F.; Ihling, C. H.; Schäfer, M.; Sinz, A. Cleavable Cross-Linker for Protein Structure Analysis: Reliable Identification of Cross-Linking Products by Tandem MS. *Anal. Chem.* **2010**, *82* (16), 6958−6968.

(12) Götze, M.; Pettelkau, J.; Fritzsche, R.; Ihling, C. H.; Schäfer, M.; Sinz, A. Automated Assignment of MS/MS Cleavable Cross-Links in Protein 3D-Structure Analysis. *J. Am. Soc. Mass Spectrom.* **2015**, *26* (1), 83−97.

(13) Arlt, C.; Götze, M.; Ihling, C. H.; Hage, C.; Schäfer, M.; Sinz, A. Integrated Workflow for Structural Proteomics Studies Based on Cross-Linking/Mass Spectrometry with an MS/MS Cleavable Cross-Linker. *Anal. Chem.* **2016**, *88* (16), 7930−7937.

(14) Liu, F.; Rijkers, D. T. S.; Post, H.; Heck, A. J. R. Proteome-Wide Profiling of Protein Assemblies by Cross-Linking Mass Spectrometry. *Nat. Methods* **2015**, *12* (12), 1179−1184.

(15) Liu, F.; Lössl, P.; Scheltema, R.; Viner, R.; Heck, A. J. R. Optimized Fragmentation Schemes and Data Analysis Strategies for Proteome-Wide Cross-Link Identification. *Nat. Commun.* **2017**, *8*, 15473.

(16) Kolbowski, L.; Mendes, M. L.; Rappsilber, J. Optimizing the Parameters Governing the Fragmentation of Cross-Linked Peptides in a Tribrid Mass Spectrometer. *Anal. Chem.* **2017**, *89* (10), 5311−5318.

(17) Giese, S. H.; Belsom, A.; Rappsilber, J. Optimized Fragmentation Regime for Diazirine Photo-Cross-Linked Peptides. *Anal. Chem.* **2016**, *88* (16), 8239−8247.

(18) Smith, D.-L.; Götze, M.; Bartolec, T. K.; Hart-Smith, G.; Wilkins, M. R. Characterization of the Interaction between Arginine Methyltransferase Hmt1 and Its Substrate Npl3: Use of Multiple Cross-Linkers, Mass Spectrometric Approaches, and Software Platforms. *Anal. Chem.* **2018**, *90* (15), 9101−9108.

(19) Deng, W.; Shi, X.; Tjian, R.; Lionnet, T.; Singer, R. H. CASFISH: CRISPR/Cas9-Mediated in Situ Labeling of Genomic Loci in Fixed Cells. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (38), 11870−11875.

(20) Rappsilber, J.; Mann, M.; Ishihama, Y. Protocol for Micro-Purification, Enrichment, Pre-Fractionation and Storage of Peptides for Proteomics Using StageTips. *Nat. Protoc.* **2007**, *2* (8), 1896−1906.

DOI: 10.1021/acs.jproteome.8b00947
J. Proteome Res. 2019, 18, 1363−1370

(21) Giese, S. H.; Fischer, L.; Rappsilber, J. A Study into the Collision-Induced Dissociation (CID) Behavior of Cross-Linked Peptides. *Mol. Cell. Proteomics* **2016**, *15* (3), 1094−1104.
(22) Vizcaíno, J. A.; Csordas, A.; del-Toro, N.; Dianes, J. A.; Griss, J.; Lavidas, I.; Mayer, G.; Perez-Riverol, Y.; Reisinger, F.; Ternent, T.; et al. 2016 Update of the PRIDE Database and Its Related Tools. *Nucleic Acids Res.* **2016**, *44* (D1), D447−D456.
(23) Kolbowski, L.; Combe, C.; Rappsilber, J. xiSPEC: Web-Based Visualization, Analysis and Sharing of Proteomics Data. *Nucleic Acids Res.* **2018**, *46* (W1), W473−W478.
(24) Bollineni, R. C.; Koehler, C. J.; Gislefoss, R. E.; Anonsen, J. H.; Thiede, B. Large-Scale Intact Glycopeptide Identification by Mascot Database Search. *Sci. Rep.* **2018**, *8* (1), 2117.
(25) Diedrich, J. K.; Pinto, A. F. M.; Yates, J. R., III. Energy Dependence of HCD on Peptide Fragmentation: Stepped Collisional Energy Finds the Sweet Spot. *J. Am. Soc. Mass Spectrom.* **2013**, *24* (11), 1690−1699.
(26) Iacobucci, C.; Piotrowski, C.; Aebersold, R.; Amaral, B. C.; Andrews, P.; Borchers, C.; Brodie, N. I.; Bruce, J. E.; Chaignepain, S.; Chavez, J. D. Cross-Linking/Mass Spectrometry: A Community-Wide, Comparative Study Towards Establishing Best Practice Guidelines. 2018, *bioRxiv* 424697. bioRxiv.org Preprint Server..
(27) Mohr, J. P.; Perumalla, P.; Chavez, J. D.; Eng, J. K.; Bruce, J. E. Mango: A General Tool for Collision Induced Dissociation-Cleavable Cross-Linked Peptide Identification. *Anal. Chem.* **2018**, *90* (10), 6028−6034.
(28) Mendes, M. L.; Fischer, L.; Chen, Z. A.; Barbon, M.; O'Reilly, F. J.; Bohlke-Schneider, M.; Belsom, A.; Dau, T.; Combe, C. W.; Graham, M. An Integrated Workflow for Cross-Linking/mass Spectrometry. 2018, *bioRxiv* 355396. bioRxiv.org Preprint Server..
(29) Gutierrez, C. B.; Yu, C.; Novitsky, E. J.; Huszagh, A. S.; Rychnovsky, S. D.; Huang, L. Developing an Acidic Residue Reactive and Sulfoxide-Containing MS-Cleavable Homobifunctional Cross-Linker for Probing Protein−Protein Interactions. *Anal. Chem.* **2016**, *88* (16), 8315−8322.
(30) Gutierrez, C. B.; Block, S. A.; Yu, C.; Soohoo, S. M.; Huszagh, A. S.; Rychnovsky, S. D.; Huang, L. Development of a Novel Sulfoxide-Containing MS-Cleavable Homobifunctional Cysteine-Reactive Cross-Linker for Studying Protein-Protein Interactions. *Anal. Chem.* **2018**, *90* (12), 7600−7607.
(31) Burke, A. M.; Kandur, W.; Novitsky, E. J.; Kaake, R. M.; Yu, C.; Kao, A.; Vellucci, D.; Huang, L.; Rychnovsky, S. D. Synthesis of Two New Enrichable and MS-Cleavable Cross-Linkers to Define Protein-Protein Interactions by Mass Spectrometry. *Org. Biomol. Chem.* **2015**, *13* (17), 5030−5037.

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J. Proteome Res. 2019, 18, 1363−1370