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REVIEW

Progress in methodologies and quality-control strategies in protein cross-linking mass spectrometry

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Abstract

Deciphering the interaction networks and structural dynamics of proteins is pivotal to better understanding their biological functions. Cross-linking mass spectrometry (XL-MS) is a powerful and increasingly popular technology that provides information about protein-protein interactions and their structural constraints for individual proteins and multiprotein complexes on a proteome-scale. In this review, we first assess the coverage and depth of the XL-MS technique by utilizing publicly available datasets. We then delve into the progress in XL-MS experimental and computational methodologies and examine different quality-control strategies reported in the literature. Finally, we discuss the progress in XL-MS applications along with the scope for future improvements.

KEYWORDS
cross-linking, interaction network, mass spectrometry, protein-protein, proteomics

1 | INTRODUCTION

Protein-protein interactions play a central role in virtually all biological pathways [1]. Mass spectrometry-based proteomics approaches play a pivotal role in identification and quantification of protein-protein interactions [2]. Some of the most widely used mass spectrometry based strategies to study protein-protein interactions include affinity purification combined with mass spectrometry (AP-MS), proximity labeling combined with mass spectrometry [3], limited proteolysis-coupled mass spectrometry (Lip-MS) that employs a double-protease digestion step [4] and protein correlation profiling (PCP) that relies on a co-fractionation based clustering approach [5–7]. More recently, Cross-linking mass spectrometry (XL-MS) has become an increasingly popular technique that can elucidate structural restraints in addition to the detection of protein-protein interactions on a proteome-scale [8–10].

Chemical cross-linking of proteins was first demonstrated to be a very important tool to study protein-protein contacts about four decades ago [11, 12]. It was initially utilized in conjunction with techniques such as electrophoresis and X-ray crystallography [11, 13, 14]. Later, with the developments in the field of mass spectrometry, Young et al. [15] reliably identified the fold of bovine basic fibroblast growth factor (FGF)-2, providing momentum for the rapid development of the XL-MS technology [16]. More specifically, development of novel cross-linkers with diverse capabilities (such as non-cleavable and cleavable cross-linkers, homo- and hetero-multifunctional cross-linkers), various acquisition strategies and fractionation and enrichment methods have been reported. Additionally, the downstream computational pipelines for reliable identification and analysis of the experimental data are shown to be equally important for the robustness of the XL-MS

ABBREVIATIONS: XL-MS, cross-linking mass spectrometry; PDB, protein data bank; NHS ester, N-hydroxysuccinimide ester; ABAS, azido-benzoic-acid-succinimide; Azide-A-DSBSO, azide-tagged, acid-cleavable disuccinimidyl bis-sulfoxide; Alkyne-A-DSBSO, alkyne-tagged, acid-cleavable disuccinimidyl bis-sulfoxide; BDRG, biotin aspartate rink glycine; BMSO, bismaleimide sulfoxide; BS2G, bis(sulfosuccinimidyl) glutarate; BS3, bs(sulfosuccinimidyl) suberate; CBDPS, cyanurbiotindipropionylsuccinimide; CBS, carboxy-benzophenone-succinimide; CDI, 1,1′-carbonyldiimidazole; DHSO, dihydrazide sulfoxide; DMDSSO, dimethyl disuccinimidyl sulfoxide; DMTMM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; DSBSO, disuccinimidyl bis-sulfoxide; DSBU, disuccinimidyl dibutyric urea; DSG, disuccinimidyl glutarate; DSS, disuccinimidyl suberate; DSSO, disuccinimidyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; ICATXL, Isotope coded and affinity tagged cross-linking; Leiker, lysine-targeted enrichable cross-linker; PIR, protein interaction reporter; SDA, succinimidyl diazirine; SDASO, succinimidyl diazirine sulfoxide

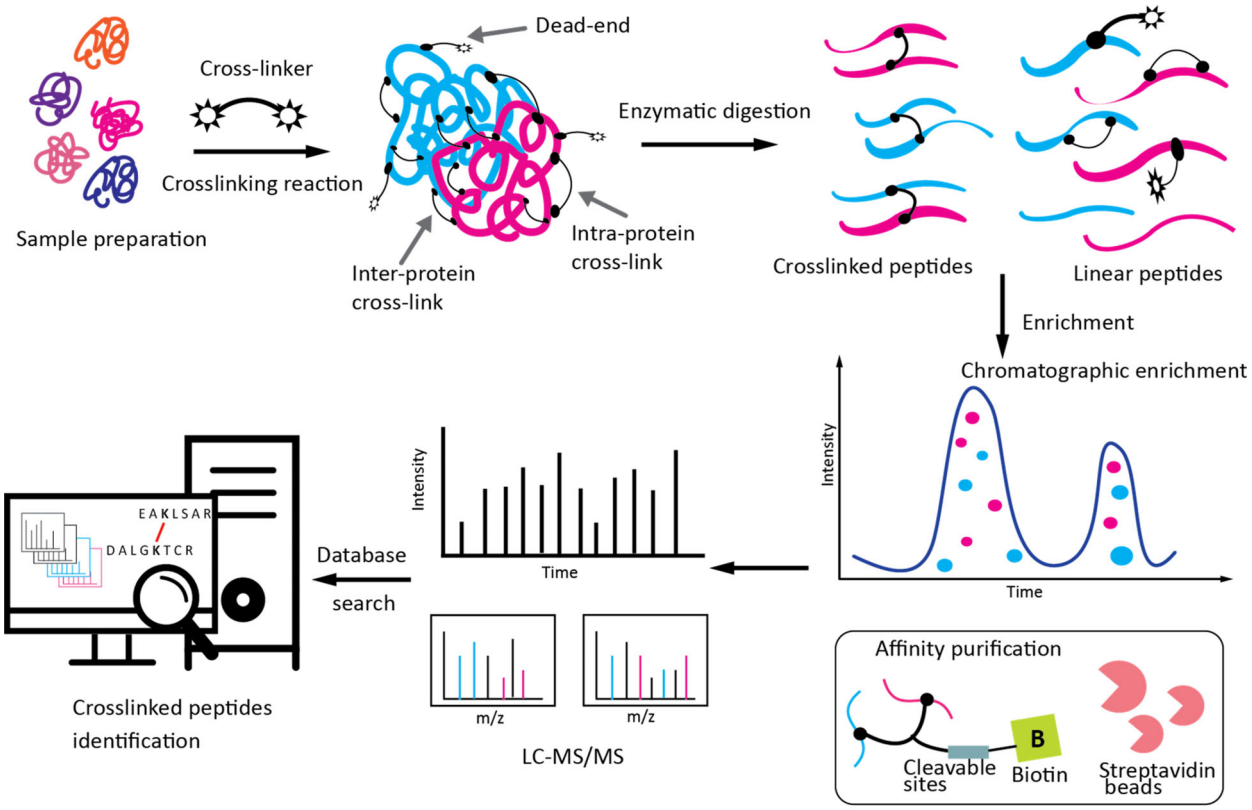

FIGURE 1 Outline of the general XL-MS workflow

technology [14]. Notably, significant improvements have been reported in the cross-link search algorithms, validation, and quality-control methodologies.

Supported by the innovative developments, XL-MS has been applied on diverse biological systems with varying complexities, ranging from single protein analyses to proteome-scale interactome studies. In this review, we assess the current state of the XL-MS technology in terms of the coverage of the published datasets and summarize the technical advancements in the experimental as well as computational methodologies along with the scope for further advancements.

2 | GENERAL WORKFLOW OF XL-MS

XL-MS has made great progress over the past few decades and the recent advancements facilitated its evolution as a well-established technology. Although the specific experimental methodologies and data analysis pipelines vary between multiple labs [8, 17, 18], the general pipeline of XL-MS typically includes choosing a sample system, cross-linking reaction, enzymatic digestion, enrichment of cross-linked peptides by chromatographic separation or affinity purification, mass spectrometric analysis, and identification of the cross-linked peptides (Figure 1). In the following sections, we discuss the advancements in the experimental and computational methodologies in XL-MS workflow, along with diverse quality-control strategies reported in the literature.

3 | COVERAGE OF XL-MS DATASETS

One of the major challenges faced by mass spectrometry based proteomic approaches is the relatively lesser coverage of the lower abundance proteins in comparison to the proteins with higher abundance in the cell [19]. Similarly, the large-scale and proteome-wide XL-MS studies tend to identify much higher fraction of cross-links from highly abundant complexes such as ribosome and proteasome compared to that of proteins with relatively lower abundance [20]. To examine this comprehensively, we compiled a set of 31,745 cross-links among 4035 proteins from published datasets [21–38] and analyzed them in relation to the cellular abundance of the cross-linked proteins, obtained from PaxDB [39] (3688 out 4035 proteins had abundance values in the PaxDB which contribute to 28,244 cross-links in the dataset) (Figure 2A, 2B). The results showed that about 76% of the cross-links map to the proteins that belong to the top 10% category in terms of their cellular abundance (Figure 2B). We further noted that only about 2% of the cross-links were mapped to proteins from the bottom 50% category (Figure 2B). These results clearly indicate the need for systematic approaches to increase the sensitivity towards the low-abundant proteins, thereby increasing the overall depth of XL-MS technology [20].

Furthermore, we examined the availability of three-dimensional structures from the PDB database [40] for the cross-linked residue pairs in the dataset and observed that about 65% of them (20,616 out of 31,745) do not have structural information (Figure 2C). We

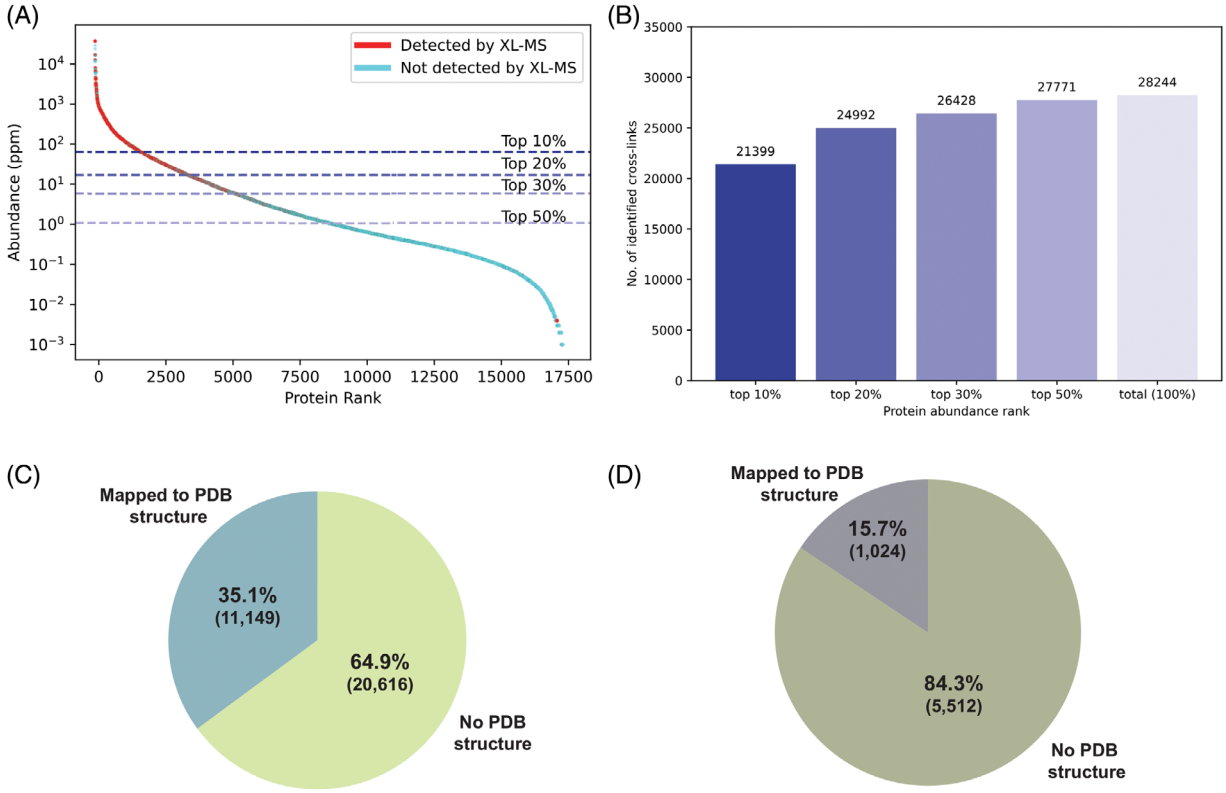

FIGURE 2 Depth of the human proteome achieved by XL-MS. (A) The abundance of the human proteome detected by XL-MS. The x-axis is the rank of the protein when ordered by abundance and the y-axis is the raw abundance. The proteins detected by XL-MS are shown as red spheres and those not detected are shown as blue spheres. (B) The distribution of cross-links across protein abundances. For example, the 20% most abundant proteins in the human proteome were involved in 24992 cross-links. (C) Proportion of cross-links (both intraprotein and interprotein) in the XL-MS dataset with or without structural information in PDB. A cross-link is considered "mapped" to a PDB structure if both residue positions involved in the cross-link are found on the same PDB structure. (D) Proportion of protein-protein interactions inferred from the inter-protein cross-links in the XL-MS dataset with or without structural information in PDB. An interaction is considered "mapped" if there exists any co-crystal structure in PDB containing those two respective proteins

also noted that only about 16% of the protein-protein interactions inferred from the XL-MS dataset have three-dimensional structures in PDB (Figure 2D). These observations emphasize that XL-MS provides invaluable structural information, complementing the existing structural biology techniques. Additionally, we assessed the coverage of the XL-MS dataset in terms of the functional biological complexes from CORUM database [41]. We observed that 1305 out of the 2417 human complexes had at least one interprotein cross-link mapped to at least one of their subunits, and 1055 of them had about one-third of their subunits with one or more interprotein cross-links mapped (Table 1). Overall, these analyses suggest a reasonable coverage for functional biological complexes and a significant opportunity for further improvements.

TABLE 1 Coverage of biological protein complexes by XL-MS

Fraction of subunits covered	Covered by	
	Interprotein Cross-links	All Cross-links
> 0%	1305	1833
≥ 20%	1194	1780
≥ 25%	1084	1712
≥ 30%	1055	1696
≥ 35%	766	1348
≥ 40%	707	1293
≥ 45%	690	1276
≥ 50%	391	848

4 | PROGRESS IN EXPERIMENTAL METHODOLOGIES

In this section, we discuss the key components of experimental methodologies of XL-MS workflow namely cross-linkers, fractionation methods, along with quantitative XL-MS.

4.1 | Cross-linkers

In chemical cross-linking experiments, cross-linking reagents consisting of two or more reactive groups joined by a spacer arm are utilized to create covalent linkages between the reactive functional groups (such as primary amines) of amino acid residues that are in close proximity.

TABLE 2 Summary of some of the widely used cross-linkers for biological studies

Cross-linker	Cleavable	Residue reactivity	Spacer length	Enrichment handle	Commercial availability	Reference
BS3	No	Lys-Lys	11.4 Å	No	Yes	[48]
DSS	No	Lys-Lys	11.4 Å	No	Yes	[49]
Leiker	No	Lys-Lys	9.6 Å	Yes	No	[50]
PhoX	No	Lys-Lys	5 Å	Yes	No	[43]
DMTMM	No	Lys-Glu/Asp	0 Å	No	Yes	[51]
EDC	No	Lys-GLU/Asp	0 Å	No	Yes	[52]
SDA	No	Photo-Lys	5 Å	No	Yes	[53]
DSSO	Yes	Lys-Lys	10.1 Å	No	Yes	[54]
DSBU	Yes	Lys-Lys	12.5 Å	No	Yes	[55]
CBDPS	Yes	Lys-Lys	14 Å	Yes	Yes	[56]
PIR	Yes	Lys-Lys	43 Å	Yes	No	[57–59]
CDI	Yes	Lys-Lys	2.6 Å	No	Yes	[60]
DSBSO	Yes	Lys-Lys	14 Å	Yes	Yes	[61, 62]
DHSO	Yes	Glu/Asp-Glu/Asp	12.4 Å	No	Yes	[63]
BMSO	Yes	Cys-Cys	24.2 Å	No	Yes	[64]

The distance constraints between the cross-linked residues are defined by the length of the spacer arm, which in turn provides low resolution structural information pertaining to the interaction topologies and the tertiary structure of proteins. Typically, the distance constraint for a given cross-linker can be estimated by utilizing molecular dynamics simulation based approaches [42, 43].

The concept of protein cross-linking was reported nearly six decades ago. In 1958, Zahn et al. developed the first cross-linker using a Sanger's reagent derivative 1,5-difluoro-2,4-dinitrobenzene on insulin [44, 45]. Over the past few decades, researchers in this field have designed and developed a slew of cross-linkers, providing structural biologists and systems biologists with a wide variety of tools [46, 47]. Generally, cross-linking reagents can be classified into two categories based the presence (cleavable linkers) or absence (non-cleavable linkers) of labile bonds (summarized in Table 2). The labile bonds that are cleaved within the mass spectrometer upon collisional energy including the collision-induced dissociation energy (CID) and higher-energy collisional dissociation (HCD) or electron-transfer fragmentation (ETD), helps uncouple cross-linked peptides in MS2. The resulting peptide pair modified by the cleaved cross-linkers can be separately sequenced in MS3, thereby facilitating efficient cross-link identification.

4.1.1 | Non-cleavable cross-linkers

Two of the most widely used non-cleavable cross-linkers in proteomic studies include DSS [48] and BS3 [49], which were developed almost four decades ago. Their utility in diverse range of XL-MS studies has been demonstrated in a recent community-wide study [17, 47]. The chemical structures of DSS and BS3 are similar, and the main difference lies in the reactive group that targets the primary amine. More specifically, DSS carries a classical NHS ester, whereas BS3 carries a water-soluble sulfo-NHS ester. The fragmentation pattern of the DSS cross-linker is depicted in Figure 3A. Due to the commercial availability and well-established experimental and computational pipelines, DSS, and BS3 have been widely applied to study purified proteins, protein complexes [65–67], organelles [68, 69], intact cells [70] and both isotope-labelled [69, 71, 72] and label-free quantitation [73, 74].

The variants of DSS and BS3 with different spacer arm, for example, DSG and BS2G, have also been widely employed [72, 75]. Specifically, the length of the spacer arm in case of DSG and BS2G 7.7 Å, which is shorter than that of DSS and BS3 (11.4Å), helps in obtaining complementary structural information. Furthermore, to capture higher resolution distance restraints, "zero-length" cross-linkers such as EDC [52] and DMTMM [51] have been utilized. Moreover, the data analysis pipelines established for other non-cleavable cross-linkers could be utilized for the protein samples cross-linked by EDC and DMTMM [51, 76, 77].

A class of hetero-bifunctional non-cleavable cross-linking reagents has also been reported. Their reactive groups constitute a lysine-reactive NHS-ester in combination with a residue-unspecific photoreactive group [53], such as aryl azide (azido-benzoic-acid-succinimide (ABAS)), diazirine (succinimidyl-diazirine (SDA)) and benzophenone (carboxy-benzophenone-succinimide (CBS)). Furthermore, homo-bifunctional and hetero-bifunctional cysteine-specific cross-linkers have also been developed [78, 79]. Although these cysteine-specific linkers provide additional information and complementary spatial distance constraints to the homo-bifunctional NHS-ester cross-linkers, they are not hugely popular due to the relatively low abundance of cysteine residues in proteins.

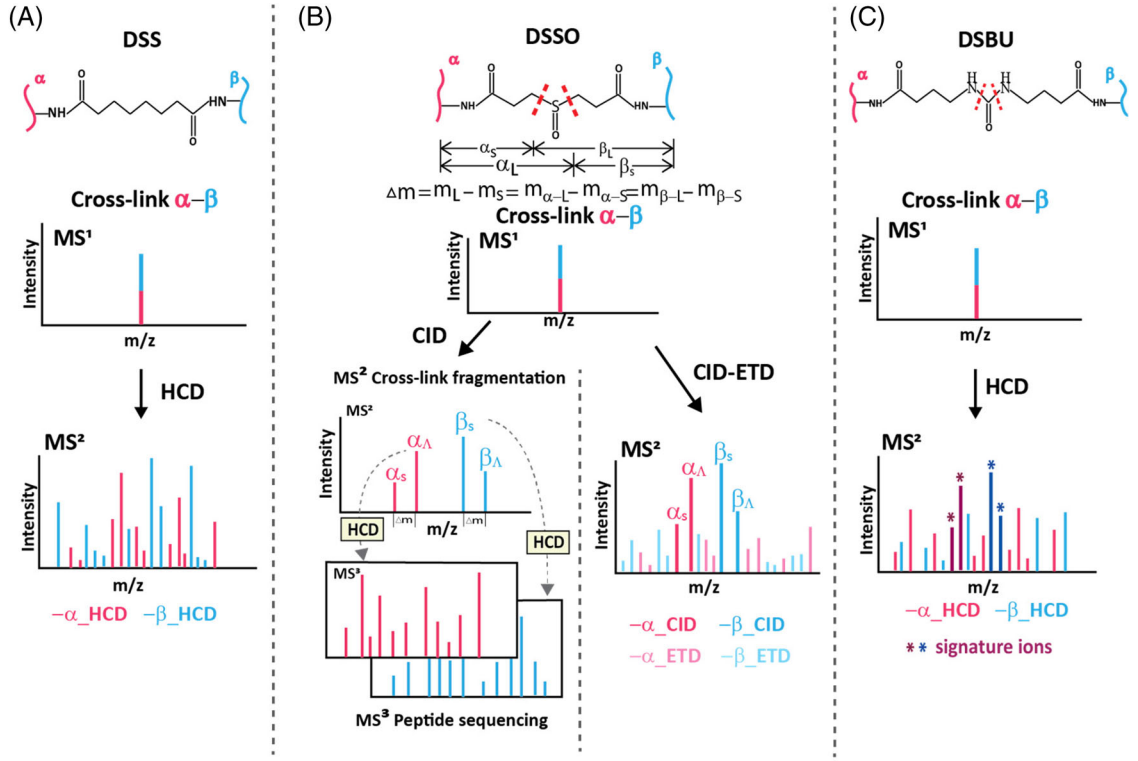

FIGURE 3 Fragmentation patterns of representative non-cleavable and cleavable cross-linkers. (A) DSS, (B) DSSO and (C) DSBU

4.1.2 | Cleavable cross-linkers

Despite being utilized in wide range of applications in vitro and in vivo, the non-cleavable cross-linkers face a major limitation in their applicability to large-scale and whole-proteome studies. This limitation is mainly due to the quadratic expansion of the computational search space, known as "the n^2 problem", which also increases the chance of random hits and leads to an unfavorable impact on the confidence in cross-links assignment [80]. Cleavable cross-linkers (e.g. DSSO, DSBU, PIR etc.), have been developed to address this issue. Cleavable cross-linkers contain labile bonds within the spacer arm regions that can be cleaved by photo- [57], chemical- [81] and MS energy [54-56, 58, 82–90]. After cleavage, the resulting cleaved peptides can be separated either prior to or during the MS processing. MS-cleavable cross-linkers are fragmented in the mass spectrometer and upon MS2 fragmentation of cross-link parent ions, their signature fragment ions are generated. This drastically reduces the database size because they can be searched as regular linear peptides modified by cleaved cross-linker. Leveraging this unique feature of characteristic cross-link fragments in MS2 (theoretically, the sum of MS2 fragments plus the mass of connected cross-linkers can be matched to the parent ions in MS1) addresses the n^2 problem and extensively simplifies the database searching and identification of the cross-linked peptides [80, 91]. In addition, accounting for the evidence from MS1, MS2, and MS3 levels further reduces the false positive rate.

Huang lab developed the DSSO linker [54], which is one of the most widely used commercially available cleavable cross-linker for XL-MS. For DSSO, either of the two symmetric C-S bonds near the sulfoxide moiety can be cleaved by a low CID energy at MS2 level, leading to the physical separation of the cross-linked peptides and producing unique linear peptide fragment pairs with a defined mass difference (Figure 3B). The peptide fragments are modified with cleaved cross-linker remnant moieties, an alkene or an unsaturated thiol moiety; therefore, the mass difference of the cross-linker remnant moieties from the same peptide is 31.97 Da, which is often utilized as a filter to select the qualified peptide pairs from MS2 to MS3 peptide sequencing. The advantage of such feature is that it can save time, improve searching speed and accuracy of cross-linked peptides identification through searching the conventional database, and reduce the search space from n^2 to 2n. Given the feasibility and efficiency of sulfoxide-incorporating MS-cleavable cross-linker, Huang's group has designed a series of such linkers, including DMDSSO [92], DSBSO [61], DHSO [63], BMSO [64], and SDASO [82] and demonstrated the robustness of MS-labile C-S bonds for MS2-MS3 analysis.

DSBU is another commonly used MS-cleavable cross-linker which has been developed by Sinz lab [55], based on a urea moiety carrying two symmetric cleavable C-N bonds around the central urea group (Figure 3C). However, the labile bonds of DSBU require higher cleavage energy than sulfoxide bonds of DSSO. Diagnostic fragments of DSBU generated with MS1/MS2 assist database searching by their MeroX software [93, 94]. More recently, a commercially available compound, CDI (1,1'-carbonyldiimidazole), has been reported by Sinz lab [60]. CDI contains two symmetric urea-type MS-cleavable bonds with a very short spacer arm (~2.6 Å) and can generate

characteristic fragments utilized for cross-link identification. For CDI, the frequency of cross-linking an amine to an amine is similar to that of cross-linking an amine to a hydroxy group. In addition to the cross-linkers targeting lysine, Sinz lab also developed the first MS-cleavable, photo-thiol-reactive cross-linker, 1,3-diallylurea (DAU) [95]. DAU is a commercially available reagent efficiently undergoing an anti-Markovnikov hydrothiolation with cysteine residues in the presence of UV-A irradiation. A urea group located in the spacer arm enables the cleavability of cross-linked products with collisional activation.

PIR (protein interaction reporter) cross-linkers, designed by Bruce lab, employ dual Rink structure [83] or Asp-Pro peptide bonds [59] as labile bonds that are cleaved in the mass spectrometry or through photoactivation upon electrospray ionization. To improve the identification efficiency of cross-links by PIR and enable the large-scale applications of PIR cross-linkers, a new mass spectrometry method, named ReACT (real-time analysis for cross-linked peptide technology) was developed based on unique features of PIR cross-linkers. With the incorporation of the biotin handle, PIR cross-linkers have been successfully utilized for a myriad of large-scale cross-linking applications including bacteria [32, 59, 96–99], mice [100, 101], and human systems [58, 102].

4.2 | Cross-link enrichment strategies

In spite of several advancements, some bottlenecks still exist in XL-MS such as the low abundance of cross-linked peptides vs. the linear peptides in complex peptide mixtures, and the cross-linking bias towards highly abundant proteins in proteome-wide studies. To address these limitations, researchers employ prefractionation methods at protein or peptide level in conjunction with XL-MS.

After the cross-linking reaction, reactive residues in spatial proximity remain connected and the digested mixtures include the intraprotein or interprotein cross-linked peptides, dead-end modified peptides and mono-loop modified peptides. Compared to linear peptides (containing un-modified peptides, dead-end modified and mono-loop peptides), the cross-linked peptides typically have higher charges and larger size. Taking advantage of these differences, cross-linked peptides are commonly separated from linear peptides by size exclusion chromatography (SEC) [103, 104] and strong-cation exchange (SCX) [105]. The SCX fractionation method can further be customized using StageTips [106]. Other fractionation methods such as hydrophilic strong anionic exchange (hSAX) chromatography [70] and hydrophilic interaction chromatography (HILIC) [91], which are commonly employed to increase the depth of proteome coverage and phospho-peptides enrichment [107], can also be utilized for enrichment cross-linked peptides [38, 108]. Furthermore, two-dimensional peptide separation has been shown to be efficient in recent studies [70, 109, 110]. To further improve the efficiency of SCX separation, charge-based fractional diagonal chromatography (XL-ChaFRADIC) integrating two dimensional SCX separations has been reported [111]. Additionally, different combinations of gel filtration, SCX and SEC have been explored for proteome-scale XL-MS studies [70, 112].

Affinity-based purification strategies targeting the cross-linked peptides hold great potential to boost the sensitivity of XL-MS studies drastically. The most common approach incorporates a biotin tag into the cross-linkers by either synthesizing the biotin directly within the cross-linkers or conjugating the biotin at a later step using click chemistry. Next, the cross-linked peptides are enriched by streptavidin/avidin beads, followed by a thorough washing step to remove non-specific peptides. Throughout the years, multiple cross-linkers that facilitate such enrichment have been developed, including Azide/Alkyne-A-DSBSO [61, 62], Leiker [50], ICATXL [113], CBDPS [56], BDRG [88] and cliXlink [114]. Among them, Azide/Alkyne-A-DSBSO incorporates acid cleavable sites by click chemistry into the cross-linkers and Leikers integrates an azobenzene-based cleavable site or a photo-cleavable site, by which biotin can be removed prior to MS analysis and alleviate the interference of biotin in the MS analysis of cross-linked peptides. A more recently developed cross-linker named PhoX incorporates a phosphonic acid into a non-cleavable cross-linking reagent and functions as a mimic of the phosphate group. [43]. PhoX demonstrates that the well-established phosphopeptide enrichment strategies such as IMAC could be applied for enrichment of the cross-linked peptides. The study also reported more than 1100 cross-linked sites by employing PhoX to cross-link a human cell lysate followed by IMAC enrichment and a single 180 min MS run [43].

In addition to the typical enrichment methods prior to mass spectrometry, ion mobility spectrometry can be utilized to fractionate peptides in the gas phase, offering another dimension of separation [115, 116]. Ions can be separated based on their physiochemical properties such as mass, shape, dipole moment, and charge, which has been shown to lower the percentage of linear peptides [117–119]. Recently, the application of a new generation ion mobility device, high-field asymmetric waveform ion mobility spectrometry (FAIMS), has been reported to have a similar performance on cross-link identification with SEC [120].

4.3 | Quantitative cross-linking mass spectrometry

Combining cross-linking and quantitative MS is an increasingly popular variant of XL-MS. The nature of the proteins and protein complexes within cells is dynamic in time and space. Hence, to monitor the dynamic of interactions and structure, quantitative cross-linking MS strategies are necessary to enable comparative analysis under different physiological conditions. Majority of the cross-linking MS data is generally acquired using data-dependent acquisition (DDA) methodology, routinely following trypsin digestion, ionized and analyzed by mass spectrometry. Although it is widely used and highly powerful, this methodology can be biased towards selecting peptides with the strongest signal, therefore making the quantification of low-abundance peptides challenging. About two decades ago, data-independent acquisition (DIA) concept was brought up to overcome the existing limitations of DDA. In a DIA analysis, peptides within a

defined mass-to-charge (m/z) window are selected to further fragmentation until all m/z in that range are analyzed. This leads to accurate peptide quantification with no limitation to profiling predefined interested peptides [121]. Rappsilber group applied DIA to QCLMS and found that DIA was indeed able to improve QCLMS[37, 122]. For a broader application of DIA in the future, development of robust data analysis tools for efficient processing of the highly convoluted spectral data is required.

4.3.1 | Isotope-coded cross-linkers and metabolic labeling

Similar to the stable isotope-labeling with amino acids in cell culture (SILAC) labeling, incorporation of stable isotope (e.g., ^{2}H, ^{13}C) into the cross-linkers can be employed for quantitative comparative analyses. One of the many widely used strategies is to take advantage of isotope-coded cross-linkers (e.g., d0/d4-BS3 or d0/d12-DSS). This strategy was initially explored by Rappsilber [71] and Robinson groups [123]. Utilizing the d0/d4-BS3 system, Robinson group compared the chemical cross-linking profile of untreated and dephosphorylated forms of F-type ATPase and highlighted the key role of phosphorylation in regulating nucleotide binding and stability of the chloroplast F-type ATPase. It is important to note that although d0/d4-BS3 and d0/d12-DSS are widely used and commercially available, d0/d10-DMDSSO by Huang group [58], d0/d8-BDH-NHP by Bruce group [96] and d0/d6-Leiker by Lei group [50] have all been reported to have similar utility. Software tools such as tract, MaxQuant and pQuant have capabilities to process and analyze the isotope-coded XL-MS data.

Even though the SILAC technique has been widely utilized in quantitative proteomics for almost two decades, the integration of SILAC and XL-MS has been only reported by the Bruce group [27, 102]. Bruce lab reported some of the efficient applications of SILAC in conjunction with XL-MS [27, 102]. Specifically, they compared the protein expression levels for topoisomerase-2A (TOP2A) between drug resistant and sensitive cell lines cross-linked by BDP-NHP and observed a correlation between TOP2A activity and the abundance of a cross-link spanning the DNA binding pocket of TOP2A [27]. In another work, utilizing the SILAC labeled cells treated with inhibitors of heat shock 90 (Hsp90) and PIR cross-linker, the authors captured the dynamic conformation and interaction changes of Hsp90 machinery [102]. These studies demonstrated that the combination of SILAC and XL-MS could detect functionally relevant conformational changes of proteins and protein complexes, which are helpful to deepen our understanding of the complex biological mechanisms.

5 | PROGRESS IN COMPUTATIONAL METHODOLOGIES

In this section, we summarize and discuss recent progress in the development of robust cross-link search tools and review the innovative quality control and assessment strategies reported in the literature.

5.1 | Cross-link search algorithms

It is well-known that the downstream computational tools for identification of cross-links from the raw experimental data are imperative for the cross-linking mass spectrometry technology as a whole. The task of identification of cross-linked peptides faces challenges that are far more difficult than those that are faced by single peptide identification [14]. Some of those challenges include the increased complexity of cross-linked samples [16], very high complexity of the computational search space [14, 26, 105] and relatively lower precursor intensities for the cross-links [38, 124]. A variety of cross-linkers have been utilized in XL-MS studies which include some of the most widely used chemical cross-linkers [125]. Having a robust and amenable cross-link search tool in hand is as crucial as utilizing an efficient linker molecule for any XL-MS study. Table 3 summarizes some of the recent and most commonly used cross-link search software.

Cross-link search algorithms that are designed to identify cross-links from samples cross-linked using non-cleavable linkers needs to account for all combinations of peptide pairs for the given precursor mass leading to the n^2 problem [26]. Such challenge makes it virtually impossible to perform a cross-link search on complex proteomes such as human. However, some approaches have aimed at addressing this issue by implementing an open search strategy [129, 132, 134, 139] and better optimizing the scoring functions [131]. The cleavable cross-linkers specifically addresses the n^2 problem by generating peaks with signature mass difference in the MS2 spectra [26, 55, 140] and signature fragment ions [141]. The inception of cleavable cross-linkers led to the development of various fragmentation and acquisition strategies [30] as well as corresponding software tools for cross-link identification [30, 32, 38, 128, 132, 135, 139].

5.2 | Quality control strategies

The false discovery rate (FDR) estimation from conventional proteomics studies has been adapted for cross-linking mass spectrometry [21]. The FDR for identification of linear peptides in the conventional proteomics is estimated based on the fraction of decoy identifications in peptide spectrum matches (PSMs). However, the search procedure in XL-MS is relatively complex and constitutes the consolidation of identifications at four more levels in addition to the PSMs, namely, cross-link spectrum matches (CSMs), peptide pairs, residue pairs and protein pairs. Fischer and Rappsilber [142] systematically assessed the effects of FDR estimation at different levels. They observed that controlling for FDR at just the PSM and CSM levels could result in much higher error rates at the residue pair and protein pair levels. Additionally, Beveridge et al.[163] [developed a synthetic peptide library that could be used as a ground truth, and showed that the FDR estimated by different search algorithms could often be higher than the actual underlying FDR. The importance of error estimation at different stages of cross-link identification has also been noted by other recent studies in the field [38, 143]. Moreover, Trnka et al. [139] suggested that the estimation of FDR for intraprotein and interprotein

TABLE 3 Summary of various available search tools for cross-linking mass spectrometry

Software	Web address	Amenable for cleavable linkers	Additional notes	Reference
MS-Bridge (Protein Prospector)	https://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form = msbridgestandard	Yes	Available as a web-based application	[126]
MeroX	http://www.stavrox.com/	Yes	Has been recently updated to version MeroX 2.0 [127]	[128]
Kojak	http://www.kojak-ms.org/	No	Precompiled binary formats are provided for Windows and Linux operating systems	[129]
SIM-XL	http://patternlabforproteomics.org/sim-xl/	No	Utilizes reporter ions to identify the XL spectra	[130]
XlinkX	https://www.hecklab.com/software/xlinkx/	Yes	Implemented in Proteome Discoverer software	[30]
ECL2	http://bioinformatics.ust.hk/ecl2.html	No	Utilizes an optimized scoring function	[131]
MetaMorpheusXL	https://github.com/smith-chem-wisc/MetaMorpheus	Yes	Utilizes an ion indexing algorithm for the search	[132]
Mango	https://brucelab.gs.washington.edu/software.html	Yes	Can be used in conjunction with Comet [133]	[32]
pLink2	http://pfind.ict.ac.cn/software/pLink/	No	Utilizes a two-stage open search strategy	[134]
xiSEARCH	https://www.rappsilberlab.org/software/xisearch/	Yes	FDR needs to be estimated using xiFDR tool (https://www.rappsilberlab.org/software/xifdr/)	[135]
MaXLinker	https://www.yulab.org/resources/MaxLinker/	Yes	Implements an 'MS3-centric' search algorithm [162]	[38]
LinX	https://ms-utils.org/LinX/	No	Enables identification of intermolecular cross-links from homodimer interfaces using 15N-labelling	[137]
MS Annika	https://ms.imp.ac.at/index.php?action = ms-annika	Yes	Available as a proteome-discoverer node	[138]

cross-links separately would facilitate error estimation with better reliability. More recently, Lenz et al.[143] showed that the cross-links need to be merged into protein-protein interactions for reliable estimation of error. Additionally, Keller et al.[144] established that false positive cross-links are more likely to be interprotein than intraprotein and proposed a revised FDR metric to account for the fraction of interprotein cross-links in large-scale studies. Similarly, Bartolec et al.[145] optimized their search criteria by comparing the scores of potentially true positive and false positive interprotein cross-links. Recently, de Jong et al.[146] proposed a composite filter for minimizing the error rate by utilizing the mass and charge distribution patterns in relation with elution time of the cross-linked peptides.

A widely used approach utilizes the available three-dimensional structures for validating cross-links identified from XL-MS studies. Specifically, the cross-links are mapped onto existing three dimensional structures of highly abundant complexes (such as ribosome and proteasome) and the mapped cross-links that comply with the Euclidean distance constraint of the cross-linker are considered as true positives and the ones that exceed the constraint are considered as potential false positives with some of them likely capturing the conformational changes. While this approach may provide useful insights into the quality of the cross-links and protein flexibility for studies on specific proteins and complexes, it fails to capture the true fraction of false positives for proteome-wide datasets. The main reason is that the false positive and true positive cross-links in these large-scale studies are not equally likely to fully map to an existing 3D structure, thereby leading to massive underestimation of false positives. We demonstrated this limitation using published datasets in our recent study, and we further proposed a set of four complementary validation metrics to address the issue [147]. More specifically, our proposed framework accounts for both fully and partially mapped cross-links to the structure (fraction of structure-corroborating identifications (FSI)), utilizes additional search spaces (fraction of misidentifications (FMI)), leverages known protein-protein interactions (fraction of interprotein XLs from known interactions (FKI)) and employs an orthogonal experimental assay to validate the novel interactions from the dataset.

6 | KEY APPLICATIONS OF CROSS-LINKING MASS SPECTROMETRY

One of the key goals of XL-MS is to elucidate the architecture of proteins and protein complexes and serve as a complementary approach for protein-protein interaction detection techniques such as yeast two-hybrid (Y2H), AP-MS, and proximity labeling assays as well as for classical structural biology techniques such as nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography, and cryo-EM. Some of the important applications are discussed below.

6.1 | Large-scale identification of protein-protein interactions

While the traditional methods for investigating protein-protein interactions on proteome-scale are well-established and widely utilized, they face limitations such as time, their labor intensive nature and their inability to capture transient and weak interactions. The advancements in different aspects of XL-MS discussed in the previous sections pave the way for XL-MS to become a promising tool to circumvent those limitations and capture protein-protein interactions at proteome-scale.

Multiple studies in the literature have reported an impressive number of cross-links utilizing XL-MS on cell lysates. With the optimized fragmentation method and their updated software XlinkX 2.0, Heck group identified 1158 and 3301 cross-links from *E. coli* and HeLa cell lysates, respectively [148]. Our previous study on K562 cell lysates cross-linked by DSSO reported 9319 cross-links by employing our "MS3-centric" MaXLinker search engine [38]. In another study, Sinz group performed XL-MS on Drosophila embryo extracts using DSBU and detected 7436 cross-links [149]. Apart from the lysate cross-linking studies, XL-MS has also been successfully applied on intact cells [58, 62, 70, 151, 150]. Furthermore, some studies isolated subcellular organelles to capture protein-protein interactions within the organelle and increase the depth of XL-MS identifications [68, 69, 100, 101, 153, 152]. In one such study, Rappsilber group isolated mitochondria from human K562 cells and performed in situ cross-linking with DSS coupled with sequential digestion and SCX as well as SEC fractionation. In total, they identified 5518 unique cross-linked residue pairs among 792 proteins [68]. Additionally, Bruce group employed XL-MS to investigate the mechanism and interactome of a synthetic peptide SS-31, which functions to strengthen mitochondria [100].

6.2 | Elucidating the 3D structures of biological complexes

XL-MS can provide structural information for flexible protein domains which often cannot be captured by conventional techniques utilized in structural biology. Furthermore, the structural restraints provided by XL-MS can be utilized as an input for automated large-scale structural modeling studies of functional complexes [154-156], also in conjunction with techniques such as Cryo-EM [157-159]. One of such pioneering studies successfully combined XL-MS with affinity-purification and cryo-EM to probe the interaction network of phosphatase 2A and proposed a molecular interaction model between PP2A regulatory subunit 2ABG and the TCP1 ring complex (TRiC) chaperonin [66]. A more recent study has successfully combined whole-cell XL-MS, cellular cryo-electron tomography, and integrative modeling to determine an in-cell architecture of a transcribing-translating expressome at sub-nanometer resolution [70].

7 | CONCLUSION

In this review, we discussed the advancements in different modules in the XL-MS workflow. With exciting advancements in both the fields of cross-linking (with the advent of several new cross-linkers) and mass spectrometry (mass spectrometers with superior sensitivity), cross-linking mass spectrometry has become one of the most popular techniques for dissecting protein-interactions and understanding their structural dynamics. Here, we comprehensively reviewed the developments in the experimental as well as the computational aspects of the XL-MS workflow. Moreover, as with any other high-throughput technique, quality-control and assessment are of utmost importance for XL-MS and some exciting studies have aimed at addressing the existing limitations. We summarized such quality assessment strategies reported in the literature and discussed some of the key approaches in detail. In the future, different combinations of various chromatographic fractionation methods such as SEC, SCX, and HILIC can be extensively explored to improve the coverage for proteome-scale crosslinking studies. Moreover, efficient enrichable crosslinkers will play a crucial role in future for minimizing the background noise from unmodified peptides [160]. Additionally, advancing the research in the areas of cell permeable crosslinkers, cross-linking of the intact cells should propel the field forward. The existing gap between the number of known interactions and their three dimensional structures can be reliably bridged by developing robust structure modeling pipelines that can seamlessly integrate the available structure information for individual proteins (from resources such as AlphaFold database [161]) and the distance restraints from XL-MS studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study were collected from reference numbers [21–38].

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