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Publication Date

2023-10-01

DOI

10.1016/j.cbpa.2023.102357

Peer reviewed

HHS Public Access

Author manuscript

Curr Opin Chem Biol. Author manuscript; available in PMC 2024 May 14.

Published in final edited form as:

Curr Opin Chem Biol. 2023 October ; 76: 102357. doi:10.1016/j.cbpa.2023.102357.

New Advances in Cross-linking Mass Spectrometry Toward Structural Systems Biology

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Abstract

Elucidating protein-protein interaction (PPI) networks and their structural features within cells is central to understanding fundamental biology and associations of cell phenotypes with human pathologies. Owing to technological advancements during the last decade, cross-linking mass spectrometry (XL-MS) has become an enabling technology for delineating interaction landscapes of proteomes as they exist in living systems. XL-MS is unique due to its capability to simultaneously capture PPIs from native environments and uncover interaction contacts though identification of cross-linked peptides, thereby permitting the determination of both identity and connectivity of PPIs in cells. In combination with high resolution structural tools such as cryo-electron microscopy and AI-assisted prediction, XL-MS has contributed significantly to elucidating architectures of large protein assemblies. This review highlights the latest developments in XL-MS technologies and their applications in proteome-wide analysis to advance structural systems biology.

Keywords

Protein-protein interaction; cross-linking mass spectrometry; structural proteomics; integrative structural analysis; protein complexes; structural systems biology

Introduction

Protein-protein interactions (PPIs) are central to the structure and function of protein complexes. These modular assemblies work hand-in-hand to establish an intricate proteome network that defines a cell's functional states under different physiological and pathological conditions. Unsurprisingly, aberrations in PPIs and protein complex organization can have drastic impacts on basic cellular processes, and thus have been associated with a multitude of human diseases over the past several decades. Directly targeting PPIs has become an attractive strategy for therapeutics, and its clinical potential has been demonstrated by recent success in the development of 'molecular glues' that facilitate protein interactions to modulate protein degradation. Given their critical importance, systematic elucidation of PPIs with molecular and structural details in their native environment towards structural systems

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

biology has become a focal point in modern proteomics research. The information obtained will not only advance our understanding of fundamental biology and human pathologies, but also provide new targets for developing improved therapeutics.

High-resolution structures of proteins and protein complexes have been achieved through X-ray crystallography and nuclear magnetic resonance (NMR), with a sharp increase in cryo-electron microscopy (cryo-EM) owing to recent technological advancements. However, structural elucidation of compositionally and conformationally heterogenous protein complexes remains difficult with traditional biophysical methods. This has led to a rapid development of integrative approaches utilizing static structure information in conjunction with mass spectrometry (MS)-based structural methods including native MS, hydrogen-deuterium exchange, cross-linking mass spectrometry (XL-MS), surface labeling, and limited proteolysis.

Among these methodologies, cross-linking mass spectrometry (XL-MS, also abbreviated as CL-MS or CX-MS) is a powerful technology for PPI discovery and characterization. XL-MS is unique due to its capability to capture endogenous PPIs in native cellular environments by forming covalent bonds among three-dimensionally proximal residues within and between proteins by chemical cross-linking. The identified cross-linked peptides enable simultaneous determination of PPI identities and their contacts at residue-level resolution. In addition, distance restraints defined by cross-linkers have been successfully utilized to validate and refine existing protein structures, as well as for *de novo* structural modeling to elucidate architectures of large protein complexes [1–7]. Its ability to sample heterogeneous and dynamic protein complexes allows the discovery of conformational states that cannot be easily assessed from static structures obtained using conventional structural tools. As such, XL-MS is uniquely positioned to allow the delineation of intricate wiring of proteome networks with structural details at the systems-level in living organisms. The information obtained will help define the modular assemblies critical in shaping cellular states and phenotypic changes associated with human diseases. XL-MS technologies have been constantly evolving towards the goal of structural systems biology and have been extensively reviewed in recent years [1–10]. Here, we present a brief overview highlighting new advances in XL-MS methods and applications during the last two years with a special emphasis on proteome-wide studies.

Addressing Challenges in Proteome-Wide Analysis

Cross-linked peptides are often hard to detect during MS analysis due to their heterogeneity and low abundance. In addition, cross-linked peptides composed of two peptide constituents yield complex MS/MS spectra, making their unambiguous identification difficult. Both of these hindrances are magnified with increasing sample complexity and especially apparent during PPI profiling at the systems-level. To address these inherent challenges, numerous advancements in sample preparation strategies, cross-linking reagents, data acquisition and analysis have been accomplished during the last decade to make XL-MS an enabling technology for global PPI mapping *in vitro* and *in vivo* (Figure 1) [1,4,5,7–9,11]. It is noted that thousands of cross-links and PPIs have been identified from *in vivo* XL-MS studies on various sample origins including bacteria [12], mammalian cells [13–18], and tissues

[19]. Compared to *in vitro* XL-MS analyses of cell lysates, *in vivo* XL-MS experiments have resulted in the identification of considerably more inter-protein PPIs [14–18]. In comparison to molecular crowding during *in vivo* cross-linking, native cell lysis required for *in vitro* cross-linking not only dissipates subcellular compartments and changes protein concentration, but also reorganizes dynamic, transient and/or weak protein assemblies and PPI networks. Regardless, similar to any proteomics studies, global XL-MS analyses have shown a preference for abundant proteins. Only a fraction of the proteome has been uncovered by the XL-proteomes [14–22]. Clearly, new developments are needed to expand not only the depth, but also the breadth of XL-proteomes.

Enhancing the Detection of Cross-linked Peptides

Due to the high dynamic range of proteomes, enrichment of cross-linked peptides is essential to the success of proteome-wide XL-MS studies. This can be accomplished by employing cross-linkers that carry an affinity tag (e.g. biotin or phosphonic acid tag) or enrichable handle (e.g. azide/alkyne tags for click chemistry conjugation) to allow enrichment of cross-linked peptides in complex peptide mixtures (Figure 1) [12,14–16,18–20,23–25]. Interestingly, polyclonal antibodies targeting two MS-cleavable cross-linkers DSSO (Disuccinimidyl sulfoxide) and DSBU (Disuccinimidyl dibutyric urea) have been recently developed to probe cross-linked proteins [26]. While their applicability in XL-MS analysis needs to be demonstrated, the availability of cross-linker-specific antibodies presents a unique means for optimizing protein cross-linking and enriching cross-linked proteins and peptides. However, affinity-based enrichment alone is often insufficient to effectively detect the most structural informative cross-links, i.e. inter-linked peptides, in the presence of abundant linear cross-linked (i.e. dead-end (mono-link) and intra-linked (loop-linked)) peptides. Thus, peptide separation techniques such as size exclusion (SEC) [14], strong cation exchange (SCX) [18,19], and high pH reverse phase (HpH-RP) chromatography [16,20] have been employed as additional fractionation steps to further improve the detectability of cross-linked peptides. With the development of two-dimensional peptide separations (e.g. SEC-HpH-RP and SCX-HpH-RP), non-enrichable cross-linkers have been successfully applied for proteome-wide analyses to generate XL-data at a scope comparable to those using enrichable cross-linkers [21,27].

To differentiate between co-occurring protein complexes, oligomers, and conformers during large scale analysis, MS-based complexome profiling has been effectively coupled with XL-MS, allowing the determination of protein complex organization with subunit composition, subunit stoichiometry and connectivity (Figure 1) [20]. A workflow combining blue native PAGE separation with in-gel XL-MS has also been developed to augment global description of protein complexes and demonstrated on purified bovine heart mitochondria [20]. In addition, in-cell or ex vivo cross-linking has been coupled with subcellular fractionation [19,28–30] to reduce sample complexity and increase PPI mapping on specific subproteomes. In addition, protein complexes can be affinity purified after *in vivo* cross-linking or for *in vitro* cross-linking to investigate subunit organization and structural topologies [31–35]. Moreover, the feasibility of combining APEX2-based proximity labeling with lysate cross-linking has been shown in dissecting subcellular interactomes [36,37]. Taken together, integration of protein and peptide separation techniques would be beneficial

to enhance the in-depth analysis of cellular networks and the characterization of protein complexes (Figure 1).

Improving the Identification of Cross-linked Peptides

In comparison to standard bottom-up proteomic studies where MS/MS spectra of linear peptides are searched against a database of all *n* possible enzyme-generated peptides, matching of cross-linked peptide spectra requires the consideration of n^2 combinations, drastically expanding search space, computational demand, and time, as well as making the control of false discovery rate (FDR) difficult. These challenges have been previously circumvented by the development of MS-cleavable cross-linkers which enable physical separation of cross-linked peptide constituents within the mass spectrometer for subsequent MS^3-based peptide sequencing, permitting cross-link identification through linear peptide searches using conventional database searching tools (Figure 2) [1,38]. Because of the simplified and accurate identification of cross-linked peptides, MS^n-based MS-cleavable XL-MS platforms are considered advantageous, especially for global PPI mapping. This has been demonstrated by various *in vitro* [21,22] and *in vivo* [14,39] large-scale studies. However, the reduced speed and sensitivity of MS^n- compared to MS^2-only acquisitions have been suggested to be a limiting factor. Thus, alternative database search algorithms and scoring functions have been developed in recent years to permit efficient MS^2-based analysis of MS-cleavable XL data for global PPI analysis [11,40,41]. Given the benefits of MS^n and MS^2-type acquisitions [11,42], we anticipate that their integration would facilitate the expansion of XL-proteomes. While the applicability of non-cleavable cross-linkers for large scale analyses has been demonstrated [12,15–17], MS-labile reagents have proven beneficial in reducing the ambiguity of peptide identifications during MS^2 analysis [42], critical for deriving reliable interactomes. One caveat of MS^2 acquisitions is that the FDRs of intra- and inter-protein linkages need to be considered separately due to the increased likelihood of forming decoy-containing inter-subunit cross-links [43]. Furthermore, due to error propagation across different levels of XL-MS results (i.e. CSM, cross-linked peptides, residue pairs, and PPIs), FDR at each level needs to be carefully controlled [43,44]. To benchmark cross-linking search engines, synthetic peptide libraries have been developed in recent years in order to accurately estimate FDR from various XL-MS workflows [45,46].

Expanding PPI Coverages with Combinatory Approaches

Currently, lysine-reactive cross-linkers remain the most widely used reagents due to the effectiveness of amine-reactive chemistry and the high occurrence of lysines in proteins and at PPI interfaces (Figure 2). However, lysine-targeting reagents alone cannot uncover the complete map of proteome networks as numerous PPI contact regions lack lysine residues. Thus, combinatory XL-MS approaches utilizing multiple cross-linking chemistries have been applied to expand PPI coverage [1]. Recent XL-MS analyses have further demonstrated multi-chemistry complementarity for increasing PPI coverage by coupling lysine cross-linkers with carboxyl-reactive [13,20,47–51], lysine-to-cysteine [31] and cysteine [22] cross-linkers.

Interestingly, cross-linkers made of different reactive groups and/or spacer arm structures/lengths but targeting the same residues can also lead to the discovery of complementary PPIs

[1,2,20,52]. Thus, continued efforts have been made to develop new cross-linkers, notably enrichable lysine cross-linkers designed for *in vivo* XL-MS studies [12,15,16]. In addition, a new class of lysine cross-linkers based on di-ortho-phthalaldehyde (DOPA) has been recently reported [53]. In comparison to NHS (N-hydroxysuccinimide) esters, DOPA-based cross-linkers are non-hydrolyzable and reactive at low pH and temperature, presenting the possibility of analyzing PPIs in extreme conditions. More importantly, the reaction kinetics of DOPA are significantly faster (by 60~120 times), permitting cross-linking within seconds. This has been shown to be particularly beneficial for capturing transient interactions and snapshots of protein unfolding during time course experiments. While this fast chemistry prevents their use for in-cell cross-linking, the development of DOPA linkers provides a new opportunity to uncover PPIs previously inaccessible to NHS ester-based reagents. Moreover, the commonly used fixation reagent formaldehyde has been explored for XL-MS studies owing to its cell permeability and fast reaction kinetics. However, its application to PPI mapping has been challenging due to difficulty in the identification of formaldehyde cross-linked peptides resulted from complex reactive chemistry. A recent study has discovered that formaldehyde cross-linking generates predominant cross-linked products with a mass addition of 24 Da instead of conventional 12 Da adducts, permitting the identification of cross-linked peptides from mammalian cells [54]. Although successful, the number of identified PPIs is limited and the cross-linking reaction mechanism remains elusive. Thus, how to effectively identify formaldehyde cross-linked peptides for global PPI mapping requires further exploration.

While dihydrazide chemistry has been proven effective for acidic residue cross-linking, its applications in proteome-wide analysis have been limited due to low reactivity and the need for a conjugating step using zero-length cross-linkers (e.g. DMTMM) [1]. A recent comparison of three carboxyl-reactive (i.e. hydrazide, amino, and aminooxy) groups, has revealed that the latter two groups are also suited for protein cross-linking with the amino reactive group having the highest reactivity [55]. In addition, their feasibility in XL-MS analyses of *E.coli* lysates has been illustrated. To complement lysine- and acidic residue-targeting cross-linkers, the cysteine-reactive bromoacetamide-based MS-cleavable cross-linker DBrASO has been developed to enable proteome-wide XL-MS analysis [22]. In comparison to the maleimide-based cysteine-reactive MS-cleavable linker BMSO, DBrASO possesses better specificity at physiological pH and is non-hydrolyzable, thus yielding more homogenous cross-linked products to facilitate their identification. The analysis of DBrASO cross-linked HEK 293 cell lysates identified additional PPIs and increased the scope of XL-proteomes revealed by DSSO cross-linking [22].

In addition to residue-specific cross-linking chemistries, heterobifunctional cross-linkers composed of an NHS ester and a nonspecific photoactivable diazirine are valuable in probing PPI regions that are inaccessible to residue-specific cross-linkers [1,48,56,57]. To facilitate the identification of photocross-linked peptides, three sulfoxide-containing MS-cleavable NHS-diazirine cross-linkers, namely SDASO (succinimidyl diazirine sulfoxide), have been developed [56]. The MS^n-based workflow allowed effective identification of SDASO-cross-linked peptides to generate a comprehensive interaction network of the yeast 26S proteasome complementary to existing data. Recently, Faustino, et al has shown the feasibility of photocross-linking for global analysis of *E. coli* cells and lysates by developing

new heterobifunctional photo-crosslinkers utilizing an MS-labile urea group (Faustino, 2022, bioRxiv). While MS-cleavability is critical for reducing the potential combinations of cross-linked sites obtained by photo-activatable reagents, the development of software suites using novel algorithms is also critical to facilitating cross-link identification. For instance, SpotLink has been recently developed using the dual pointer dynamic pruning algorithm and efficient memory operations, permitting the identification of nonspecific cross-links obtained by non-cleavable photocross-linkers at the proteome scale [58]. Collectively, continued development of diverse cross-linker chemistries and robust cross-link search engines [59,60] remains invaluable to further boosting XL-MS technologies toward generating a complete map of interaction landscapes in cells.

Defining Interaction and Structural Dynamics with QXL-MS

In addition to defining interactome landscapes for elucidating PPI functions, XL-MS can be integrated with quantitative proteomics to determine proteome network dynamics under different conditions [1,4,61]. Similar to traditional proteomics, quantitative XL-MS (qXL-MS) strategies can be label-free but typically employ stable isotope labeling to allow pairwise or multiplexed comparisons. The relative abundances of cross-linked peptides are used to infer changes of protein interactions and conformations.

To advance qXL-MS to systems-level studies, multiplexing capability is desirable to increase throughput and decrease missing values between samples. Multiplexed qXL-MS workflows have been achieved based on the incorporation of isobaric labels into cross-linked peptides by chemical labeling (e.g. TMT (tandem mass tag)) or cross-linking reagents [1,24]. Given the potential applicability of TMT labeling to any type of cross-linked peptide regardless of cross-linker chemistry and functionality, data acquisition strategies for TMT-based multiplexed qXL-MS analysis have been further explored [62]. While MS^3-based analysis provides more accurate quantitation, it has been shown that MS^2 acquisitions utilizing stepped-HCD can be optimized for quantifying TMT-labeled DSSO cross-linked peptides [62]. This presents an adaptable qXL-MS acquisition strategy for TMT-based multiplexed quantitation of any types of cross-linked peptides. However, cautions are needed to minimize labeling variability and peptide interference during quantitation. To circumvent these potential issues, isobaric cross-linkers such as iqPIR [24] have been developed. The 6-plex MS-cleavable linker iqPIR fragments during MS^2 analysis to release higher mass reporter ions (m/z 808~826) than TMT [24] and has been successfully applied to dissect drug-induced global interactome changes in breast cancer cells [18] and failing murine hearts [19]. While successful, it can be challenging to design and synthesize isobaric cross-linkers with higher levels of multiplexing capability while maintaining ideal mass ranges of reporter ions for accurate quantitation. Regardless, these studies have paved the way to further develop multiplexed quantitation for large-scale qXL-MS studies.

In recent years, the applications of qXL-MS have been extended to study aspects of protein biology beyond simple descriptions of interaction and conformational changes, including protein activation mechanisms and binding affinities of protein complexes. Through time-resolved label-free qXL-MS, Fürsch, et al. have investigated the heat activation and client-binding modalities of sHSPs [63]. Their quantitative data have suggested a cooperative

mechanism driven by heat activation of the Hsp26 middle domain that initiates simultaneous global conformational changes within Hsp26 in the presence of its client. In addition, Hagemann, et al has developed a new qXL-MS workflow based on d_0/d_6-labeled BS2G to determine PPI interfaces and estimate the phosphorylation-dependent dissociation constants (K_D) within the kinetochore complex (Hagemann, 2022, bioRxiv). In both studies, cross-linking incubations were shortened to several minutes despite the fact that NHS ester-based reactions often require much longer durations. This suggests that faster cross-linking reactions could be beneficial for capturing specific conformational states for mechanistic understanding of protein assemblies.

XL-MS-coupled Integrative Structural Analysis

In recent years, XL-MS technology has become an integral component of integrative structural modeling approaches and established itself as the perfect partner for cryo-EM to elucidate architectures of protein complexes [3–7,33,64–66]. While a single cross-linker can produce sufficient data for integrative modeling, comprehensive cross-link data generated from combinatory XL-MS approaches based on multiple cross-linkers are beneficial for structural analysis of large protein assemblies [47,48] and for improving precision of the resulting models [67]. High-density cross-link data obtained from photocross-linking have also been shown to facilitate integrative modeling [48,57]. The complementarity of XL-MS and cryo-EM has expedited the generation of structural models that uncover molecular mechanisms underlying the function and regulation of various protein complexes, including the dihydrolipoamide succinyltransferase (E2) component of the human α-ketoglutarate dehydrogenase complex [51] and TRanscript-EXport complex [50], as well as the assembly of reovirus capsid by the prefoldin-TRiC/CCT chaperone network [64] and virus-induced remodeling of Cul4-RING ubiquitin ligase [57]. It is noted that structural insights into the exploitation of evolutionarily conserved ubiquitination machinery such as Cul4-RING ligase has the potential to improve the design of proteolysis-targeting chimera- or molecular glue-type compounds for targeted protein degradation-based therapeutics.

Recent advances of AI-based structural prediction tools such as AlphaFold2 (AF2) have begun to revolutionize the field of protein structural biology [68–71]. With over 200 million structures predicted by AF2 and 600 million by Meta AI, XL-MS stands as a critical methodology to corroborate these AI-driven models. The integration of AF2 with XL-MS has rapidly followed, not only augmenting the interpretation of cross-linking data, but also accelerating integrative structure analysis of various protein complexes including understudied ones with increased throughput. A large-scale XL-MS dataset recently generated using a combinatory DSSO, DHSO, and DMTMM approach has been used to demonstrate the potential of integrating cross-linking data with AF2-based structural prediction [13]. The resulting models of proteins and protein complexes have presented the opportunity to mine the structural proteome and interactome, revealing mechanisms underpinning protein structure and function. AlphaLink, a modified version of the AF2 algorithm, is another strategy that has been developed to explore the intersection of XL-MS and AI-based model prediction. By incorporating cross-link distance restraints to complement co-evolutionary relationships via deep learning, AlphaLink improves structure prediction to better dissect protein conformational states and dynamics in situ [28]. In

addition, integrative analysis coupling AF2 with *in situ* cross-linking has successfully resulted in a single model of the full-length SARS-CoV-2 protein Nsp2, suggesting its potential role in zinc regulation within the replication-transcription complex [34]. Moreover, the synergy of XL-MS with AI-driven modeling has been employed to define the architecture of the full-length p53 tetramer, presenting a strategy for structural elucidation of intrinsically disordered proteins (Di Ianni, 2022, bioRxiv). Structural characterization of the polymeric intraflagellar transport A (IFT-A) complex in its native environment has been carried out by combining XL-MS and AF2 with cryo-electron tomography (cryo-ET), yielding low-resolution structures of IFT-A with details on modes of associations and subunit stoichiometry in the cellular context [72]. Very recently, DSSO-based in-cell cross-linking of the model Gram-positive bacterium Bacillus subtilis with co-fractionation mass spectrometry (CoFrac-MS) and AlphaFold-Multimer has allowed the structural prediction of 153 dimeric and 14 trimeric protein assemblies, demonstrating the feasibility of assessing interaction topologies and structural features of cellular networks at a global scale [73]. Taken together, XL-MS assisted integrative structural analysis is beneficial not only for elucidating protein complex architectures, but also for determining the mechanisms underlying their function and regulation.

Conclusion

XL-MS continually proves to be unique and effective in its ability to map endogenous PPI landscapes with structural features from various sample origins including lysates, organelles, cells, and tissues. Thus, it has become the method of choice for global delineation of proteome networks to advance our understanding of native protein module topologies at the systems-level. In addition, residue-specific PPI contacts revealed by cross-link data have demonstrated crucial to integrative structural modeling for elucidating architectures of macromolecular assemblies. With the increased robustness, sensitivity, and accessibility of XL-MS technologies, their applications have been extended to mechanistic characterization of protein complexes beyond simple PPI mapping. Recent advances in sample preparation, reagent design, MS data acquisition and analysis have allowed significant expansion of the breadth and range of PPIs that can be captured. Despite this, only a fraction of proteome networks has currently been mapped. Clearly, in-depth proteome-wide PPI profiling remains technically challenging and will continue to be a focus for future XL-MS studies. Similar to conventional proteomics studies, combinations of orthogonal separation techniques at different levels including subcellular organelles, protein complexes, proteins and peptides will certainly help dig deeper in XL-proteomes. It is anticipated that global PPI profiling can be expanded to decipher intricate signaling networks with spatial and temporal resolutions under different physiological, pathological, and pharmacological conditions. While data-independent acquisition (DIA)-based qXL-MS analysis has only gained attention recently [74,75], the remarkable success of DIA-based methods in large scale proteomics will undoubtedly drive innovations in this area to enable global quantitation of cross-links with increased reproducibility, robustness, and accuracy. With the aid of cryo-ET and AI-based structural prediction tools such as AF2, 3-D description of proteome networks in cells may be realized sooner than we can imagine. Therefore, we believe that XL-MS will continue to evolve with improved capability and throughput, and the next generation will become a

part of the standard structural biologist's toolkit to advance structural systems biology and biomedical research.

AKNOWLEDGEMENTS

This work was supported by National Institutes of Health grants R35GM145249 and R01GM074830 to L.H.

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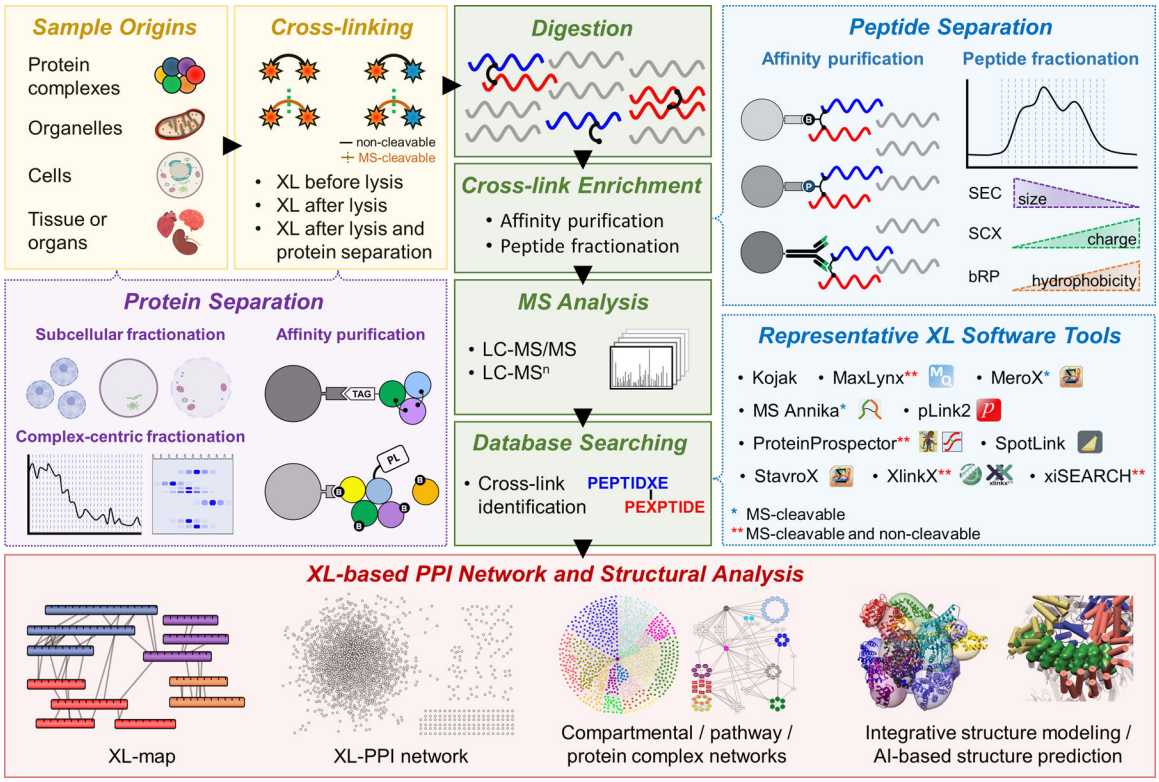

Figure 1. General XL-MS workflow.

Various sample types can be cross-linked, ranging in complexity from protein complexes to tissues and organs. Both ends of the cross-linker may target the same or different residues, while the spacer arm that connects the functional groups can be either MS-cleavable or not. To reduce complexity, proteins can be separated prior to or after cross-linking by subcellular or complex-centric fractionation, or affinity purification by tagged proteins or proximity labeling (PL). Following digestion, cross-links can be enriched by affinity purification or peptide fractionation. Cross-linked peptides can be purified if they contain a biotin or "click-able" site for appending biotin (B), phosphonic acid (P), or if an antibody recognizing the spacer arm of a cross-linker is used. Various chromatographic methods such as size-exclusion (SEC), strong-cation exchange (SCX), and high-PH reverse phase (bRP) can be used to reduce the complexity of cross-linked peptide samples prior to LC-MS analysis. Depending on the MS acquisition type (MS/MS or MS^n) and the type of cross-linker used (non-cleavable or MS-cleavable), various database search software are available to identify cross-linked peptides. Resulting cross-links can be used to generate 2-D XL-maps and XL-MS derived PPI, compartmental, pathway, and protein complex networks. Finally, cross-links can be used as distance restraints for integrative structure modeling or alongside AI-based structure prediction such as AlphaFold2 for protein structural elucidation.

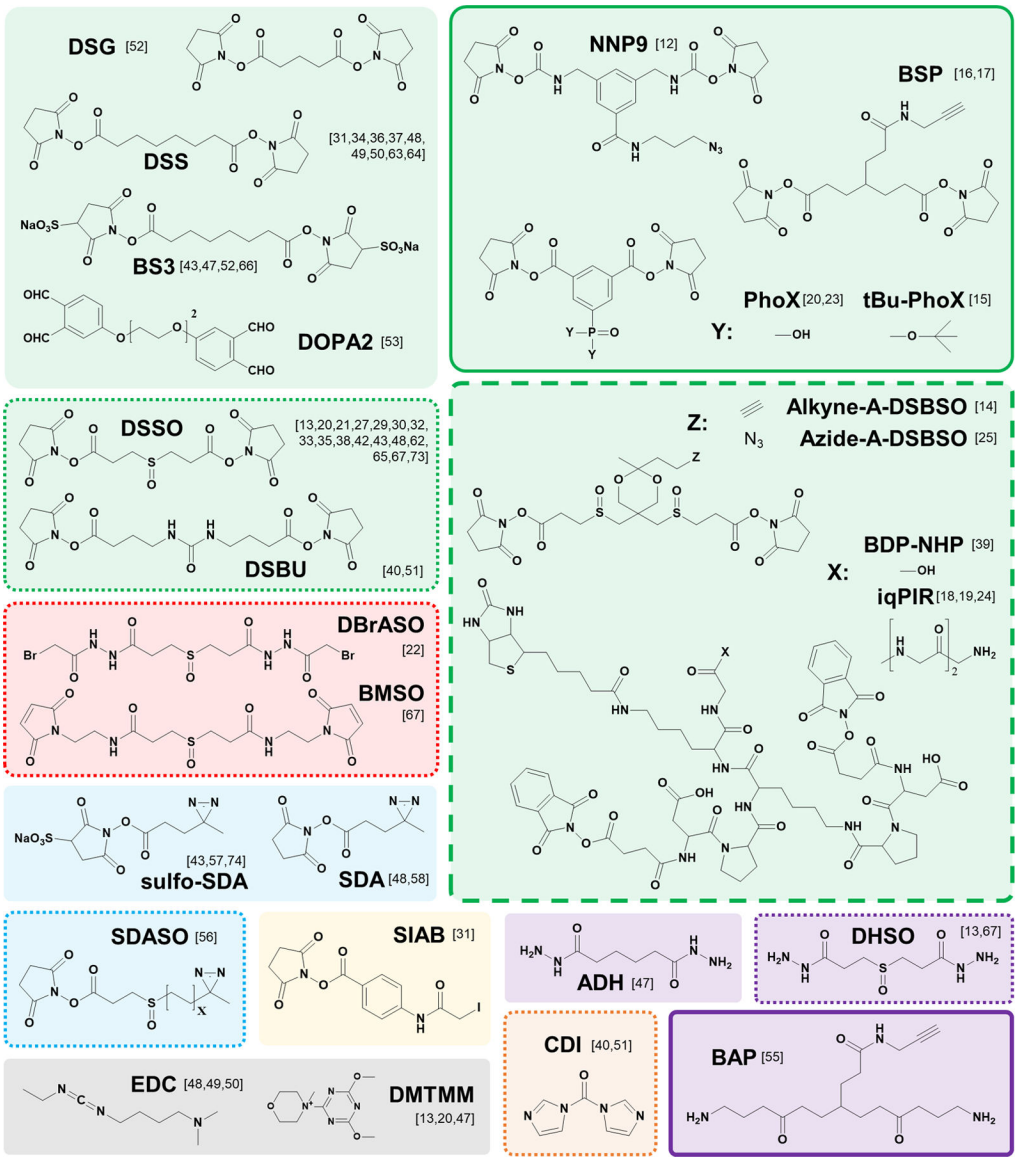

Figure 2. Selected cross-linkers discussed in this review.

Molecular structures for each cross-linker are shown alongside their corresponding references. Cross-linkers grouped and color-coded based on their targeted residues. Green: lysine-to-lysine, red: cysteine-to-cysteine, blue: lysine-to-any amino acid (nonspecific), yellow: lysine-to-cysteine, grey: lysine-to-acidic residue, orange: lysine/hydroxyl residue-to-lysine/hydroxyl residue, purple: acidic residue-to-acidic residue (requires coupling reagent such as DMTMM). The border of each group designates whether cross-linkers are MS-cleavable and/or enrichable. No border: non-cleavable and non-enrichable, thin dashed border: MS-cleavable but non-enrichable, solid border: non-cleavable but enrichable, and thick dashed border: MS-cleavable and enrichable.