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Chemical cross-linking with mass spectrometry: a tool for systems structural biology

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Biological processes supporting life are orchestrated by a highly dynamic array of protein structures and interactions comprising the interactome. Defining the interactome, visualizing how structures and interactions change and function to support life is essential to improved understanding of fundamental molecular processes, but represents a challenge unmet by any single analytical technique. Chemical cross-linking with mass spectrometry provides identification of proximal amino acid residues within proteins and protein complexes, yielding low resolution structural information. This approach has predominantly been employed to provide structural insight on isolated protein complexes, and has been particularly useful for molecules that are recalcitrant to conventional structural biology studies. Here we discuss recent developments in cross-linking and mass spectrometry technologies that are providing large-scale or systems-level interactome data with successful applications to isolated organelles, cell lysates, virus particles, intact bacterial and mammalian cultured cells and tissue samples.

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Introduction

Life relies on a vast ensemble of highly dynamic yet organized and regulated set of protein conformations and interactions to carry out biological functions. Visualizing dynamic structural and interaction changes of proteins in their native environments has the potential to greatly advance understanding of function in all areas of biology. Structural biology has primarily relied on three techniques, X-ray crystallography, NMR and Cryo-EM, to provide detailed structural information on proteins and protein complexes. Together these techniques have provided a wealth of information on the molecular structure and function of proteins, with over 140 000 structures currently deposited in the Protein Data Bank (PDB) [1]. A caveat is that these techniques generally rely on producing samples containing large amounts of highly purified proteins of interest, removed from their native environment. Furthermore, the majority of structures in the PDB are partial structures, representing static snapshots of a protein, void of any information on the conformational dynamics the protein undergoes while functioning within the living organism. Chemical cross-linking with mass spectrometry has emerged as a technique able to bridge structural biology and systems biology and provide structural information on protein complexes as they exist within a biological system.

Chemical cross-linking has long been recognized as a valuable tool for preserving the structure of biological systems through the formation of covalent bonds [2]. Several decades ago cross-linking experiments were used to provide low resolution structural information on proteins and protein complexes [3,4]. With the emergence of biological mass spectrometry came an analytical means to identify the specific cross-linked amino acid residues in a high throughput manner [5], and the combination of cross-linking with mass spectrometry (XL-MS, also CL-MS) has been established as a new technique for structural biology alongside the traditional methods [6•]. Although ultimately a relatively low resolution structural technique, the primary advantages of XL-MS are the ability to probe protein complexes of unlimited size, gain information on the native ensemble of protein conformations, and provide insight into protein structures and interactions as they exist *in vivo* [6•,7,8]. Continued advances made in recent years to mass spectrometry instrumentation, methodology and informatics now enable XL-MS to be applied to many diverse biological questions and gain new insight on the interactome. The technique of XL-MS encompasses a wide range of options that can be tailored to address a breadth of scientific enquiries. From selection from a variety of molecular cross-linkers, sample processing, LC–MS methodology and data processing and visualization tools, researchers planning to utilize XL-MS need to familiarize themselves with these options to select the best combination to suit their particular research questions. For this purpose, we refer readers to a number of recent review articles cover various aspects of XL-MS in detail that will not be covered here [6•,9–14]. This review focuses primarily on the application of XL-MS to complex samples

(i.e. intact cells, tissues, and so on) to gain structural and interaction information on a systems level.

Molecular features of chemical cross-linkers

The vast majority of XL-MS experiments carried out to date utilize homo-bifunctional cross-linking reagents that predominately react with primary amines at the ε-amino group of Lys side chain and protein N-termini. Primary amines are excellent targets for cross-linking due to their reactivity and relatively high abundance on the surface of proteins. The widespread commercial availability of N-hydroxysuccinimide (NHS) esters has made them by far the most common amine-reactive groups used in homo-bifunctional cross-linkers, although other esters including N-hydroxyphthalimide, hydroxybenzotriazole, and 1-hydroxy-7-azabenzotriazole have been used and demonstrate better reaction efficiency and kinetics [15]. Imidates are another class of amine targeting reactive groups utilized in cross-linking, however they suffer from side reactions unless the cross-linking reaction is carried out under alkaline conditions (pH > 10), limiting their use for cross-linking in physiological conditions [16,17]. This prompted Lauber et al. to utilize the suberthioimidate group as an alternative which reacts with amines at physiological pH, avoids the side reactions with imidates, and maintains the charge on the protein surface [17]. Application of the suberthioimidate cross-linker diethylsuberthioimidate (DEST) was demonstrated by Lauber *et al.* in a structural study of the *Escherichia coli* ribosome [18•]. Because suberthioimidates maintain the charge on the Lys, they can be beneficial for downstream SCX enrichment, fragmentation in the mass spectrometer and potentially less disruptive to the native protein structure making them attractive alternatives to NHS [16,17,19]. Beyond primary amines, cross-linking strategies targeting the acidic side chains in Asp and Glu have been developed. Initial demonstrations of cross-linking acidic residues utilized dihydrazide compounds which require a large excess of coupling agents to drive the reaction, limiting their usefulness for application to cellular systems [20,21]. Utilizing a double activation strategy and a diamine compound, Fioramonte *et al.* demonstrated a multiplexed cross-linking strategy linking acid groups to each other or acid groups to Lys and Ser [22]. Recently, diazo compounds been shown to cross-link acidic residues at neutral pH without the need for activating agents [23,24]. The resulting ester linkages generated from diazo based cross-linkers have also been shown to be cleavable at low collisional activation energy within the mass spectrometer, potentially making them useful for large scale acidic group cross-linking in complex cellular samples [24]. Cross-linkers targeting hydroxyl groups [25] and thiol reactive cross-linkers targeting Cys [26] have also been demonstrated and offer potential for increased depth of interactome analyses. Photo-reactive, aldehyde-based and other cross-linker chemistries which are more promiscuous in their targets, make identification of the site of cross-linking difficult and currently limit their use in structural interactome studies. Although photo-reactive cross-linkers can be utilized in combination with affinity purification mass spectrometry to identify interacting partners from complex mixtures such as cell lysates [27], without the ability to identify the specific residue sites of cross-linking limited structural information is obtained. Similarly, while formaldehyde and glutaraldehyde are some of the oldest and widely applied cross-linkers in biology and offer the beneficial characteristics of small size, high solubility, cell permeability and high reactivity the identification of the sites of cross-linking from aldehyde cross-linkers remains challenging particularly from very complex biological samples beyond model systems [28]. These issues can be overcome in part by working with relatively simple systems consisting of a small number of purified proteins, where using a combination of cross-linkers with different reactivities have found use in determining the structure of purified proteins by increasing the density of observed cross-links [29].

In addition to the variety of reactive groups, cross-linkers also contain differing spacer arm lengths which range from zero-length to tens of angstroms. The spacer arm length ultimately limits the distance between which two residues can be linked, and thus provides structural information in terms of an upper bound distance constraint. Zero-length cross-linkers provide the tightest distance restraints (generally ~10 Å Cα–C

α) as they covalently link two side chains without adding additional atoms. On the other hand, zero-length cross-linked species are generally more difficult to identify than peptides linked with longer cross-linkers that incorporate additional functionalities [30]. It would seem that choosing a smaller spacer arm length would lead to tighter restraints and thus higher resolution structural information. However, in practice it has been observed by simulation [31] and experimentally that a large percentage of cross-link distances when mapped against protein crystal structures actually exceed the theoretical spacer arm length [32]. This observation suggests that features of the protein structure itself, such as local chemical environments influencing residue reactivity, peptide backbone flexibility and conformational dynamics are more critical determinants of what residues get linked. This also explains why a cross-linker with a spacer arm of near 30 Å can produce a similar distribution of observed cross-link distances to linkers with a spacer arm near 11.4 Å, such as DSS/BS3 [33••]. In fact, cross-links exceeding the expected cross-linker distance

contain valuable information about protein dynamics representing alternative conformations from an ensemble of protein structures in solution [34]. Ultimately XL-MS is a low resolution structural technique, and while any single link may not provide a very stringent distance constraint, the combination of multiple links across the surface of a protein can significantly limit the number of possible structural models. Finally, combined use of a variety of chemical probes can offer additional advantages. As demonstrated by Brodie *et al.*, a combination of cross-linkers with differing reactive groups and spacer arm lengths provided data to better guide molecular dynamics simulations and establish protein structural models [35].

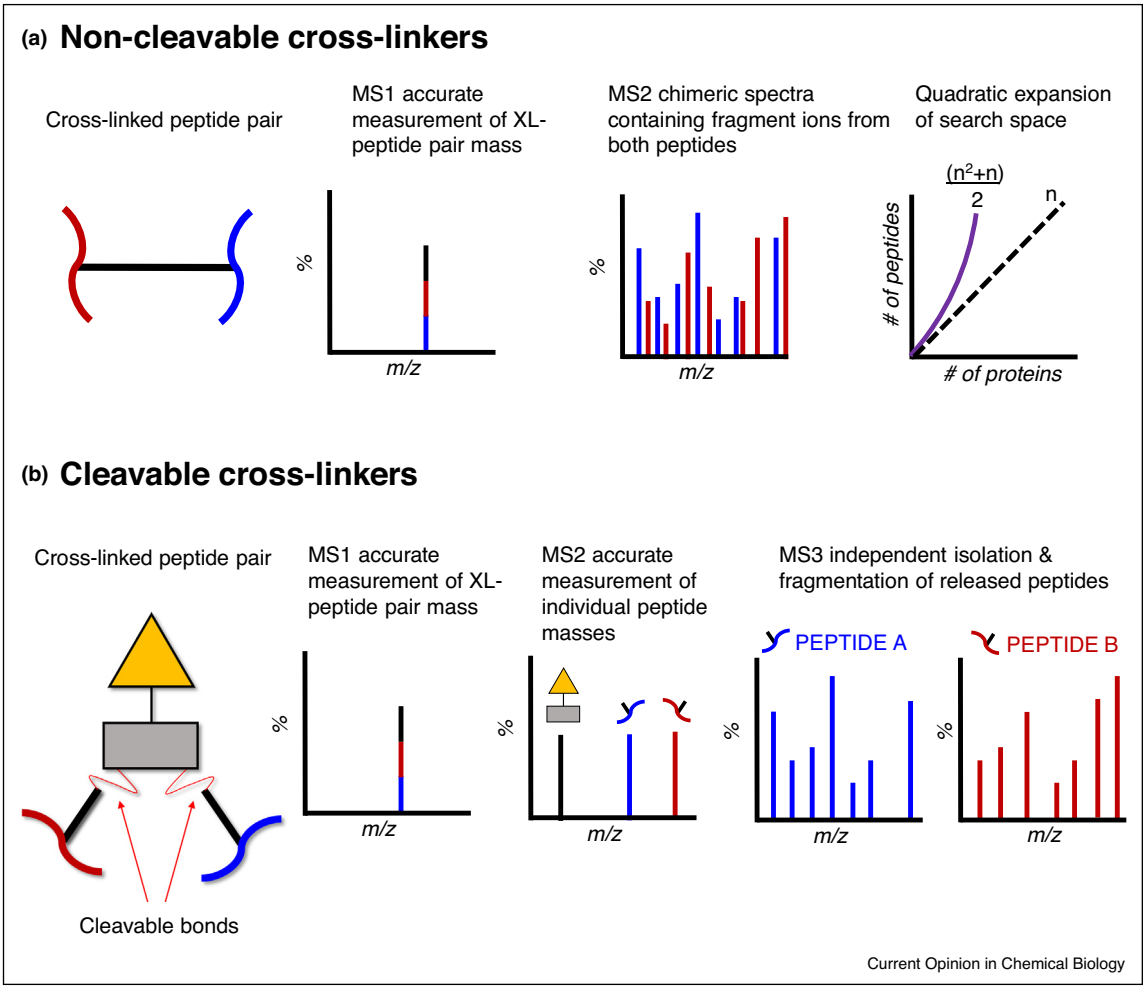
Additional features that are commonly included into the cross-linker molecular design are the incorporation of labile bonds, which are used to selectively cleave the cross-linker releasing the cross-linked molecules, affinity tags used to enrich cross-linked products from complex mixtures where the majority of components are non-cross-linked, and stable heavy isotope labels used to assist identification and/or for quantification. These features are especially important for applications of cross-linking in complex biological systems including intact cells and tissues, without which identification of the specific cross-linked amino acid residues, becomes exceedingly difficult. The most common type of labile bonds incorporated into cross-linkers are those that cleave selectively in the mass spectrometer with the input of relatively low collision induced dissociation (CID) energy (see review of cleavable cross-linkers by Sinz) [36•]. However, photocleavage has also been explored as a cross-linker cleavage mechanism [37,38] and demonstrated with interactome studies [39]. Cross-linker cleavage serves to release the two cross-linked peptides allowing their individual masses to be determined and to be independently subjected to further stages of mass spectrometry for sequence identification. Importantly this feature circumvents the combinatorial complexity that arises with the fragmentation of non-cleavable cross-linkers, and allows for unambiguous assignment of the cross-linked peptide sequences including the cross-linked sites from searching the resulting mass spectra against complete proteome databases comprised of tens of thousands of protein sequences (Figure 1). Affinity tags that have been utilized include biotin and azide functionalities and are useful for enrichment of low abundance cross-linked peptide pairs from complex sample mixtures. Affinity tags are particularly important when cross-linking intact cells and tissues as the cross-linker must permeate cell membranes and subcellular organelles to reach reactive sites on protein targets, all the while reacting with water forming hydrolyzed products of limited structural use. Therefore, the resulting inter-peptide cross-linked products, those of highest interest in identifying, are always among the lowest abundance species in the sample. The incorporation of cleavable bonds and affinity tags has proven particularly useful for large scale cross-linking efforts as are discussed below.

Fundamentally XL-MS provides multiple levels of information: the accurate masses and amino acid sequences of the cross-linked peptides, the residues that reacted with the cross-linker, and the identity of the cross-linked protein(s). It is important to note that at any of these information levels it is possible for there to exist some amount of ambiguity, redundancy and/or error to be considered in context with the scientific questions being pursued [40•]. The majority of XL-MS experiments follow a bottom-up proteomics approach, where cross-linked peptide pairs resulting from enzymatic digestion of cross-linked proteins are analyzed. See Figure 2a for a general XL-MS workflow. As cross-linked peptide pairs are generally of low abundance compared with non-cross-linked peptides, enrichment strategies including strong cation exchange chromatography (SCX) [41••], size exclusion chromatography (SEC) [42], and affinity chromatography are generally beneficial, as is sampling ions of higher charge state (4+ or greater) within the mass spectrometer [43]. Ultimately the cross-link data can be used to guide molecular modeling and docking algorithms, of which there are an increasing number of options [29,44–47], producing structural models for proteins and protein complexes. As illustrated in Figure 2b, the breadth of biological questions now addressable by XL-MS is a direct result of XL-MS technology advancement, for which specific examples are discussed next.

Cross-linking on enriched protein complexes

To date the majority of studies utilizing XL-MS have been carried out on isolated protein complexes. These studies have laid the foundation illustrating that XL-MS provides useful structural and interaction information complementary to that from traditional structural biology techniques [6•]. The addition of XL-MS into the structural biology toolbox has produced many fruitful efforts, increasing our understanding of the architecture and function of large biomolecular machines. For example, XL-MS complements cryo-EM by providing valuable information in regions of protein structures that are less well defined in cryo-EM, such as flexible regions and subunits on the periphery of a complex [48•]. In one of the first studies successfully combining XL-MS with AP-MS and cryo-EM, Herzog *et al.* probed the phosphatase 2A (PP2A) interaction network using distance restraints to propose models for interactions between PP2A regulatory proteins and the TRiC/CCT complex with its substrate 2ABG [49]. In a separate study, cryo-EM, protein tagging and XL-MS were utilized to map the locations of all subunits of the polycomb repressive

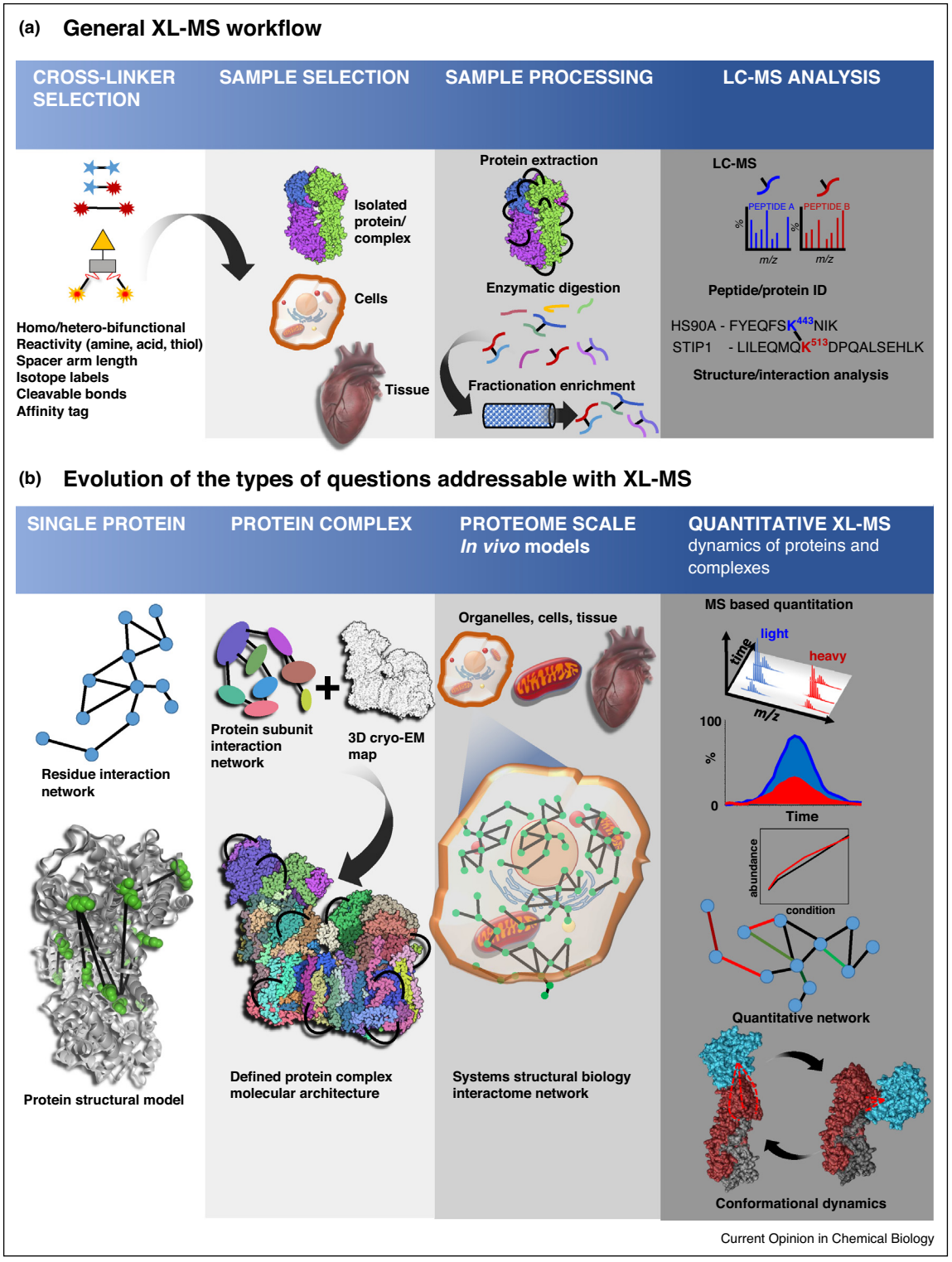
Figure 1



MS analysis of non-cleavable and cleavable cross-linked peptide pairs. **(a)** MS analysis of a peptide pair cross-linked with traditional non-cleavable cross-linkers. The MS1 spectrum provides the mass of the intact cross-linked species. Upon fragmentation in the MS2 a chimeric spectrum is generated comprised of fragment ions from both peptides and the masses of the individual peptides remains unknown. Searching MS data from non-cleavable cross-linked peptide pairs results in a quadratic expansion of the search space making proteome-wide searches an intractable problem. **(b)** MS analysis of a peptide pair cross-linked with a cleavable cross-linker. The MS1 spectrum provides the mass of the intact cross-linked species. Under MS2 the labile bonds in the cross-linker are selectively cleaved releasing the intact peptides allowing for accurate determination of their masses. Another round of MS analysis (MS3) allows for independent isolation and fragmentation of the released peptides greatly simplifying downstream analysis by circumventing the n^2 problem and allowing for proteome wide database searches to be performed.

complex 2 (PCR2) and identify interaction sites between PCR2 and its protein assembly cofactor AEBP2 [50]. A combination of cryo-EM and cross-linking were used to determine a structural model for the 2.5 MDa yeast mediator-RNA polymerase II pre-initiation complex, consisting of 52 protein subunits [51]. The nuclear pore complex (NCP) serves as an excellent example of a very large protein complex (~120 MDa in mammals) that has been successfully studied using integrative structural approaches utilizing XL-MS [52,53]. For instance, cross-links identified between Gle1 (a protein component of the mRNA export machinery) and the Nup82 holo-complex, allowed Fernandez-Martinez *et al.* to delineate the position and orientation of Gle1 within the NPC [52]. Shi *et al.* demonstrated a strategy for using XL-MS to study GFP tagged protein complexes pulled from cryoground cell and tissue powder using immobilized engineered lysineless nanobodies against GFP. The pipeline was demonstrated on three protein complexes, the yeast exosome, the anaphase-promoting complex (APC/Cyclosome)—a ubiquitin E3 ligase essential for cell cycle progression, and Beclin 1-EGFP and its binding partner Vps34 [54•]. Two recent studies have successfully used XL-MS to investigate protein interactions involved in kinetochore complexes [55,56]. Interestingly, an investigation into the impact of

Figure 2



Overview of chemical cross-linking with mass spectrometry. **(a)** General XL-MS workflow. **(b)** Types of questions that XL-MS can be used to address.

chemical cross-linking on the structures of proteins found that even at relatively high cross-linker concentration, protein folds were maintained with only minimal impact on the structure and function [57]. Empirically this observation is also supported by the high level of agreement between data from large scale cross-linking experiments with structural models in the PDB [58]. These studies, among many others, have laid a solid foundation for XL-MS as a powerful tool to investigate the assembly of protein complexes.

Proteome scale cross-linking

Mainstream structural biology techniques have traditionally required large sample amounts and high sample purity. XL-MS has the potential to overcome this limitation by being applied directly to extremely complex biological samples, including cell lysates, intact cells and tissue samples. In a pioneering study from 2008, Rinner *et al.* demonstrated the ability for identification of cross-linked peptide pairs from *E. coli* lysates with xQuest [41••]. Although ultimately limited in depth of coverage identifying cross-links in the most abundant proteins, this study demonstrated feasibility as well as the necessity for enrichment of cross-linked peptides from complex samples. In 2012 Yang *et al.* identified 394 cross-linked peptide pairs from an *E. coli* lysate and 39 from a *C. elegans* lysate [59]. In a follow up study, the same lab demonstrated the benefits of using a trifunctional cross-linker containing a biotin affinity tag (Leiker), over a traditional non-affinity tagged cross-linker (BS3), expanding the number of cross-linked peptide pairs from 394 with BS3 to 3656 pairs with Leiker in *E. coli* lysate and from 39 pairs with BS3 to 898 pairs from *C. elegans* lysate [60•]. In a XL-MS study of HeLa cell lysates and a MS-cleavable cross-linker, disuccinimidyl sulfoxide (DSSO), Liu *et al.* were able to identify over 2000 cross-linked peptide pairs including links identifying dynamic interactions between the ribosome and elongation factors [61]. In a follow up study, Liu *et al.* optimized fragmentation schemes and data analysis strategies to identify 1158 and 3301 cross-linked peptide pairs from *E. coli* and HeLa cell lysates respectively [62]. Utilizing a divide and conquer strategy with a combination of techniques along with XL-MS to probe crude subcellular fractions from the thermophilic eukaryote *C. thermophilum*, Kastritis *et al.* were able to identify 27 distinct protein communities that include 108 interconnected complexes representing a third of the proteome [63••]. A limitation of the current state of large scale XL-MS in complex samples remains the relatively small depth of coverage compared with the extreme dynamic range of protein complex concentrations in the samples. Subcellular fractionation was found to be beneficial for increasing the depth of large scale cross-linking studies carried out in *E. coli*, and *C. thermophilum* lysates [60•,63••]. Although these studies and others have demonstrated that XL-MS is applicable to samples as complex as cell lysates, applications of XL-MS to proteins as they reside within their native environments before lysis offers additional insight but presents further challenges.

In vivo cross-linking

A critical advantage of XL-MS is the ability to be applied *in vivo*, where intracellular protein concentrations can reach several hundred milligrams per milliliter and macromolecular crowding and subcellular compartmentalization exert effects on protein structures, functions and interactions [64]. Furthermore, natural co-factors, lipid membranes and interaction partners are all present during *in vivo* cross-linking at their natural physiological levels. Although *in vivo* cross-linking with aldehyde-based, photo-reactive cross-linkers, and others stabilize protein structures and interactions and can be used for identification of interacting proteins, complications with identification of the specific cross-linked residues, as discussed above in the section on cross-linker reactivity, limits structural insight from these studies. Identification of the sites of cross-linking from *in vivo* studies is enabled by utilizing cross-linkers with selectively cleavable labile bonds as well as affinity tags. Thus, the information obtained from *in vivo* XL-MS utilizing affinity tagged, cleavable cross-linkers provides insight into the structure/function relationship of proteins within living systems in a way not easily achieved with other structural biology techniques. As described below, XL-MS is generally applicable to a wide range of biological systems of extreme complexity, from infectious virus particles to intact cultured bacterial and mammalian cells to tissue samples. Thus, XL-MS is a new tool to probe protein conformations and interactions directly within the living systems under study.

Virus

Viruses are among the most basic replicating biological organisms and virion particles are composed of a small amount of genetic material either DNA or RNA embedded within a protein coat or capsid. Viruses pose an interesting subject for XL-MS as they rely exclusively on the structure and function of a relatively small subset of highly specialized proteins to carry out their replication cycle. These proteins facilitate attachment and invasion of host cells, uncoating and replication of genetic material, as well as the assembly and release of newly produced virions into the environment. XL-MS is able to provide virology researchers information on the assembly of virons, interactions with host proteins, and so on. Singh *et al.* used XL-MS to generate a pseudo-atomic model of the capsid shell of bacteriophage lambda [65]. In collaboration with researchers at the USDA, BTI and Cornell University our lab has applied XL-MS in several studies to resolve structural features and host–pathogen protein interactions of *Polerovirus* [66–68].

Bacterial cells

XL-MS has been successfully applied to study protein structures and interactions in a wide range of bacterial species including *S. oneidensis* [69], *E. coli* [43], *P. aeruginosa* [70], and *A. baumannii* [71] providing new information on membrane protein complexes such as OmcA-MtrC, the Sec translocon, and outer membrane porin proteins such as CarO with the antibiotic hydrolyzing beta-lactamase Oxa23. These efforts improved understanding of novel electron transport mechanisms and revealed a new bacterial strategy involving porin localized toxin inactivation to confer increased antibiotic resistance. Although the above studies were carried out on intact living cells, it is still necessary to remove the cells from their growth media to avoid interference with the cross-linking reaction. In a proof of concept study, using a minimal growth media with N-acetylglucosamine as the only source of energy, de Jong *et al.* demonstrated the feasibility of cross-linking a gram positive bacteria *Bacillus subtilis* in growth culture media [72].

Mammalian cells

Kaake *et al.* cross-linked HEK293 cells using membrane-permeable, enrichable, and MS-cleavable cross-linker azide-A-DSBSO (azide-tagged, acid-cleavable disuccinimidyl bis-sulfoxide) and click chemistry to couple a biotin group [73]. In total 240 non-redundant cross-linked peptide pairs were identified. They also demonstrated the ability to cross-link intact cells followed by lysis and affinity purification of histidine-biotin (HB)-tagged [74] proteasome subunits leading to increased depth of coverage of cross-links for the proteasome [75]. Cross-linking of HeLa cells with a protein interaction reporter (PIR) cross-linker revealed cross-links from all major subcellular compartments including membrane, cytosolic, nuclear, and mitochondrial proteins [76]. *In vivo* applications of XL-MS also offer a unique opportunity to study interspecies protein interactions. During invasion of human epithelial cells by *Acinetobacter baumannii*, the bacterial outer membrane protein, OmpA, a previously identified virulence factor, was cross-linked to host mitochondrial, nuclear and desmosomal proteins [77•]. Additional applications to mammalian cells are discussed in the following sections covering subcellular fractions, tissues and quantitative cross-linking.

XL-MS can also be used to identify cross-linked peptides carrying post-translational modifications (PTMs) [76]. PTMs are key regulators of protein structure and function. Although a range of proteomics techniques have emerged for the global identification and quantitation of PTMs [78], few studies have provided insight into the downstream structural and interaction changes induced by these modifications. XL-MS is uniquely suited to provide information on this level. Multiple cross-linked peptide pairs were identified providing structural information on the highly disordered histone tails modified by PTMs such as methylation and acetylation [76]. This represents an intriguing avenue for future XL-MS studies to reveal the impact on protein structures and interactions as a result of modulation of various PTM levels on a proteome-wide scale.

Cross-linking beyond intact cells, subcellular fractions, organelles and tissues

A limitation of XL-MS application to intact cells or cell lysates is that resultant data may represent an ensemble of protein conformations and interactions. Differences that may exist in each subcellular location may be blurred. So far there are a few examples of extending the principle of subcellular fractionation by applying XL-MS to intact organelles, such as mitochondria. These organelle function as the primary energy plants for eukaryotic cellular systems, in addition to regulating other key cellular processes including apoptosis, cell signaling, calcium homeostasis, and cellular proliferation. Due to the increasing realization of the importance of mitochondria in human diseases and the aging process, there is intense interest in studying the structures and interactions of mitochondrial protein complexes. Applying XL-MS to intact mitochondria isolated from bovine heart with disuccinimidyl gluterate (DSG), Wu *et al.* identified cross-links between malate dehydrogenase, citrate synthase and aconitase providing structural evidence for TCA cycle metabolon [79•]. Applying PIR cross-linking to isolated functional mitochondria, cross-linked peptide pairs from more than 300 mitochondrial proteins were identified, including links identifying mitochondrial OXPHOS supercomplexes and links in the MICOS complex [80]. Cross-links identified between ATP synthase subunits demonstrated that distinct rotational states within this molecular machine could be captured by XL-MS [80]. Similarly, Liu *et al.* were able to identify 3322 cross-linked peptide pairs from isolated mitochondria, including 4 proteins not included in mitocarta and OXPHOS supercomplex links [81]. Fasci *et al.* applied XL-MS to intact isolated nuclei and were able to visualize hot spots of interaction on nucleosomes, and utilize the distance information to build low resolution models for several nucleosome interacting proteins including Ran GTPase and the high mobility group N protein (HMGN2) [82]. Together these studies illustrate the promise of probing the proteome organization in subcellular organelles with XL-MS. Recently the ability to use XL-MS to derive large scale structural information on proteins from mouse heart tissue was demonstrated [83•]. Beyond analysis of cross-links from the whole tissue extract, it was shown that mitochondria could be isolated post-tissue cross-linking and analyzed to increase the depth of coverage for this organelle. With the expansion to tissues and the ability to fractionate subcellular compartments either before or post cross-linking, XL-MS appears well situated to provide unprecedented details into the spatiotemporal organization of the proteome.

Quantitative cross-linking and applications to cellular systems

Beyond the identification of proximal residues in proteins, the extension of traditional quantitative proteomics methods with chemical cross-linking can provide information on the dynamics of protein structures and protein complexes. There have now been a number of studies applying qXL-MS on purified protein systems and proteome wide studies to gain valuable insight into the conformational and interaction dynamics of proteins and protein complexes. Multiple studies have utilized deuterium isotope labeled cross-linkers for quantitative purposes including those by Fischer *et al.* using HSA [84] and in a series of studies of conformational dynamics of the compliment protein C3 [85–87]. Illustrating the potential for qXL-MS to probe the effects of PTMs, Schmidt *et al.* used isotope labeled BS3 to quantify phosphorylation induced conformational changes in chloroplast ATP synthase [88]. Additionally qXL-MS can probe structural changes due to ligand binding as demonstrated by Kukacka *et al.* who compared the effects of calcium binding on the conformation of calmodulin [89]. Incorporation of deuterium labels into a PIR cross-linker allowed for large scale qXL-MS in *E. coli* cells [90]. Despite these successful examples use of deuterium as an isotopic label introduces some complications into the quantitative analysis. For one, spectral complexity is increased by splitting the signal from each cross-linked peptide pair into light and heavy isotope channels. Another complication is that deuterium shifts the retention time of the labeled analyte, complicating peak detection and integration analysis. In an interesting proof of concept study, TMT isobaric mass tags were used to quantify differentially mixed samples of DSSO cross-linked cytochrome C [91•]. This suggests future studies could take advantage of the multiplexing ability of isobaric mass tags to conduct comparative qXL-MS studies across multiple conditions within a single LC–MS run.

Avoiding the use of isotopic labeling entirely, label free MS1 based quantitation (LFQ) of cross-linked peptide pairs has also been successfully applied and shown to be as reproducible as traditional quantitative proteomics methods [92,93]. The performance of LFQ was also found to be similar to using isotope labeled cross-linkers as Walzthoeni *et al.* demonstrated using both approaches to detect conformational changes in two model systems: firefly luciferase and bovine TRIC complex [94•]. Although LFQ has yet to be applied in a large scale cross-linking experiment it could be useful in addition or as an alternative to using isotope labels as in traditional quantitative proteomics experiments [95].

The metabolic incorporation of isotope labels using SILAC [96], allows for large scale *in vivo* qXL-MS measurements to be performed in cultured cells as demonstrated in a comparison between chemo-resistant and sensitive cancer cells [97]. Use of SILAC has the added benefit of allowing for global protein expression level changes to also be measured from the same sample by quantifying the non-cross-linked peptides. The benefit of having both protein level and cross-link level quantitation is demonstrated by the following example. Despite no change in transcript or protein expression levels for topoisomerase-2-A (TOP2A) between the drug resistant and sensitive cell lines, an increase in a cross-link spanning the DNA binding pocket of TOP2A correlated with increased TOP2A activity and resistance to the active metabolite of the topoisomerase inhibitor irinotecan [97]. Thus, qXL-MS offers the opportunity to detect functionally-relevant protein conformational changes that can impact understanding of complex biological mechanisms like acquired chemoresistance yet may be difficult to identify with any other large-scale measurement.

Beyond binary comparisons between different phenotypes, qXL-MS has also been applied to detect drug induced changes to protein structures and interactions in cells. By treating SILAC labeled HeLa cells with different types and concentrations of heat shock 90 (Hsp90) inhibitors, dynamic conformational and interaction changes to *in vivo* Hsp90 machinery were detected [33••]. This work is of particular interest here as it demonstrated that quantitative measurements on cross-linked peptide pairs can scale with drug concentration and can be used to distinguish different protein conformational changes induced by treating cells with different classes of small molecule inhibitors.

An interesting aspect arising from large scale qXL-MS experiments is that only a small subset of cross-linked peptide pairs display differential levels depending on the conditions under comparison. Thus, once the masses and sequences for these pairs are known they can be subjected to targeted quantitative mass spectrometric assays such as selected reaction monitoring (SRM) or parallel reaction monitoring (PRM), which comprise the current gold standard for traditional quantitative proteomics methods [98]. The feasibility to utilize PRM assays for qXL-MS was demonstrated in a cross-laboratory study with students attending the Cold Spring Harbor proteomics course [99]. Using the widely distributed quantitative software package Skyline [100] to analyze and share qXL-MS data, online databases of identified cross-linked peptide pairs such as XLinkDB [58] and ProXL [101], can become useful sources of information for biologists to build assays to probe the conformations and interactions for proteins of interest akin to how the SRM atlas is for traditional quantitative proteomics [102].

Conclusion

Understanding the dynamics of protein conformations and interactions is a fundamental goal in many biological studies yet remains challenging due to interactome

complexity and limitations of available technologies. XL-MS provides a new dimension of information not readily available with established structural biology techniques. This includes structural and interaction information from purified complexes of unlimited size, as well as unique insight into the organization of the *in vivo* interactome. As such, XL-MS has the potential to bridge the gap between structural and systems biology by providing new insight into the complex molecular machinery that supports life. On the other hand, despite great progress made in recent years, currently XL-MS studies are still only scratching the surface of the complexity of the interactome. Continued developments will undoubtedly increase the depth and coverage providing researchers with valuable new knowledge to address increasingly challenging questions and improve understanding of biological systems. Particularly, advancements in sample preparation including increased fractionation, use of orthogonal separation techniques, and improved enrichment strategies for cross-linked peptides and proteins will increase the level of output from XL-MS studies. The increased sensitivity and sequencing speed of new mass spectrometric instrumentation will also benefit in this area. Quantitative XL-MS applications will play an increasingly important role in visualizing protein structural and interaction dynamics, and will necessitate development of new informatics strategies to process, visualize and interpret the quantitative data in structural terms. Targeted LC–MS methods, such as PRM, will be employed on a larger scale to monitor specific protein conformations and interactions of interest under varying perturbations or phenotypic differences. Overall the potential of XL-MS is only beginning to be revealed for large scale systems structural biology measurements.

Conflict of interest

Nothing declared.

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- • of special interest
- •• of outstanding interest

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