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Interrogating the architecture of protein assemblies and protein interaction networks by cross-linking mass spectrometry

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Proteins are involved in almost all processes of the living cell. They are organized through extensive networks of interaction, by tightly bound macromolecular assemblies or more transiently via signaling nodes. Therefore, revealing the architecture of protein complexes and protein interaction networks is crucial to understand their function. Towards this aim, cross-linking mass spectrometry (XL-MS), which allows the elucidation of structures and interactions of proteins at low-resolution, has emerged as a valuable technology. Especially in recent years, the substantial development of cross-linking approaches and MS-based technologies, has led to noteworthy advances in the analysis of purified/*in vitro* reconstituted very large (megaDa) protein assemblies and various endogenous protein complexes in cells. Here we review the advances of XL-MS technologies and highlight some of the most recent studies. They clearly indicate that current XL-MS methodologies are ideally positioned to bridge the gap between proteomic-based interactome studies and high-resolution structural biology-based technologies.

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Introduction

Proteins, the key biomolecular players in almost all cellular processes, are commonly organized into well-regulated pathways, networks, and/or stable macromolecular assemblies, through protein–protein interactions. In recent years, chemical cross-linking combined with mass spectrometry (XL-MS) has become an increasingly powerful approach to study protein interactions, whereby the information of both the connectivity and the structures of proteins/protein assemblies can be simultaneously obtained. Cross-linking reagents are small molecules, typically containing two reactive groups that allow covalent linking of amino acid residues. After proteolytic digestion of the protein mixture, the cross-linked residues are identified by mass spectrometry. Since the two amino acid residues need to be spatially in close proximity to be conjugated, the cross-link imposes a distance constraint on the protein assembly. This distance constraint, which is defined by the sum of the cross-linker spacer arm length and the side chain lengths of the two linked residues, is essential for structural analysis and identification of protein–protein interactions [1–5].

During the last decade, XL-MS has become a very attractive complementary method to the more conventional structural biology approaches [1–5]. Compared to high-resolution methods such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, which still work the best for highly purified and/or over-expressed recombinant protein complexes, XL-MS can be performed more directly under near-physiological conditions requiring only modest amounts of sample. Although XL-MS has achieved considerable success by providing low-resolution structural information on purified proteins and protein complexes, the full realization of its power has only become apparent in the last few years. Helped by advances in cross-linking chemistry, sample preparation, cross-link enrichment, MS technology, and tools for data analysis, XL-MS has been recently applied to a wide range of studies, including the characterization of structures and/or interactions of single proteins [6], protein complexes [7–9], large macromolecular assemblies [10–13,14•,15–23,24•], and heterogeneous mixtures of protein complexes [25••,26,27]. Moreover, studies have also been performed to decipher protein structure and interactions in whole cell lysates or even in intact cells [28,29••,30••,31–34].

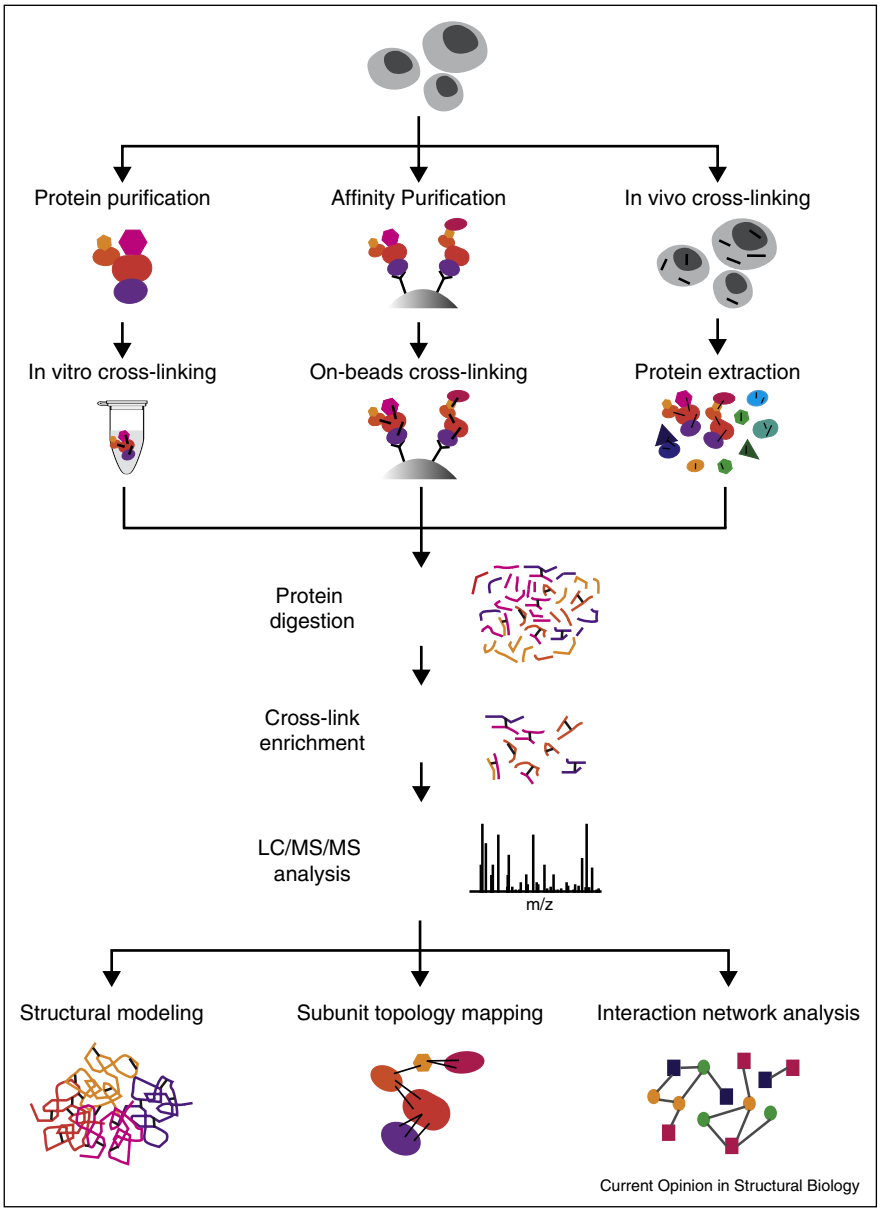
In this review, we first discuss recent developments and remaining challenges with respect to state-of-the-art cross-linking workflows, including the design of cross-linking reagents, sample preparation strategies and XL-MS dedicated bioinformatics tools. Next, we highlight certain recent XL-MS studies on *in vitro* reconstituted or endogenously purified large macromolecular protein assemblies, affinity purified protein complex mixtures and progress in proteome-wide cross-linking approaches in cells or cell lysates.

Recent improvements in the XL-MS workflow

The majority of XL-MS studies follow a generic workflow, whereby the cross-linked proteins are first digested, where after cross-linked residues are identified at the peptide level. The typical procedure of a XL-MS experiment consists of the following consecutive steps: purification of the target protein complexes, chemical cross-linking, enzymatic digestion into peptides, separation and/or enrichment of the cross-linked peptides, mass spectrometry analysis, and database searching (Figure 1). The distance information obtained from these experiments can be further subjected to various applications, such as structural modeling, subunit topology mapping and protein interaction network analysis. Although seemingly straightforward, XL-MS studies are still often considered challenging primarily due to two major obstacles: (1) the complexity of the peptide mixture resulting from the proteolytic digestion and (2) the difficulties in data analysis.

Considering sample preparation, the proteolytic digested peptide mixture of cross-linked proteins consists of

Figure 1



The generic workflow of XL-MS. XL-MS can be applied to endogenously purified/*in vitro* reconstituted proteins/protein assemblies, affinity-purified protein complex mixtures (on-beads cross-linking) and intact cells (*in vivo* cross-linking). Cross-linked proteins are digested to peptides, and thereafter the cross-linked peptides are enriched and subsequently analyzed by LC/MS. The maximal distance constraints of cross-links can be applied to structural modeling, subunit topology mapping and protein interaction network analysis.

unmodified peptides, dead-end modified peptides (peptide attached with one-end hydrolyzed cross-linker), intra-peptide cross-links (single peptide connected by the cross-linker) and inter-peptide cross-links (two peptides connected by the cross-linker). A more detailed discussion about the nomenclature of cross-linking products has been previously described [35]. Unfortunately, the most informative inter-peptide cross-links are typically of lower abundance compared to the large excess of other types of peptides in the mixture, especially the unmodified peptides. To obtain more useful information from cross-linking experiments, the enrichment of the lower abundant inter-peptide cross-links is one of the key prerequisites. Based on different chemical and physical properties between cross-linked and linear peptides, cross-linked peptides can be enriched by strong cation exchange (SCX) [36] or size exclusion chromatography (SEC) [37]. Alternatively, affinity chromatography can also be used for the enrichment of peptides modified with affinity-tagged cross-linkers, such as biotin-tagged cross-linkers that allow the enrichment using avidin-beads or columns [28,38]. Furthermore, azide-tagged or alkyne-tagged cross-linkers can be used to capture the cross-linked peptides using click-chemistry-type reactions [34,39,40].

The main bottleneck of data analysis algorithms for XL-MS is a computational challenge known as 'the n-square problem'. Inter-peptide cross-links are two linear peptides covalently linked together, whereby the precursor mass obtained from the MS scan is the summed mass of the two linked peptide moieties and the cross-linker spacer arm. Therefore, the search engine needs to consider all the possible peptide pair combinations from the linear peptide database, which dramatically increases the search space. To address the 'n-square problem', efforts have been directed to the development of new search algorithms and the design of novel cross-linkers. The search algorithm xQuest [29••,31] incorporates a pre-filter step to match cross-linked precursors and particular fragment ions by using isotopic-coded cross-linkers, to reduce the number of permutations that needs to be considered. Alternatively, algorithms such as pLink [30••], Protein Prospector [41] and MXDB [42] consider cross-linked peptide as a single peptide bearing an unknown and large mass modification, which can then be searched against a regular linear peptide database. Another attempt to tackle the 'n-square problem' is the introduction of MS-cleavable cross-linkers [28,34,43–45]. This type of cross-linker incorporates preferential gas-phase cleavable site(s) in the linker region, producing pairs of linear peptides (with part of the linker attached) upon gas-phase fragmentation during MS^2 events. Subsequently, the separation of inter-peptide cross-links in MS^2 permits MS^3 analysis of each linked peptide simplifying interpretation and identification. Very recently, algorithms that directly identify cross-links from MS^2 spectra of MS-cleavable cross-linkers were described. For instance, the cross-link search engine XlinkX, developed in our laboratory, is able to both determine the accurate precursor masses and obtain the sequences of the two linked peptides from MS^2 spectra only [46••]. Compared to MS^3-based approaches, MS^2-based methods substantially increase the instrument duty cycle and the product ion intensity, establishing a faster and more accurate high-throughput data acquisition and analysis pipeline for cross-linking studies.

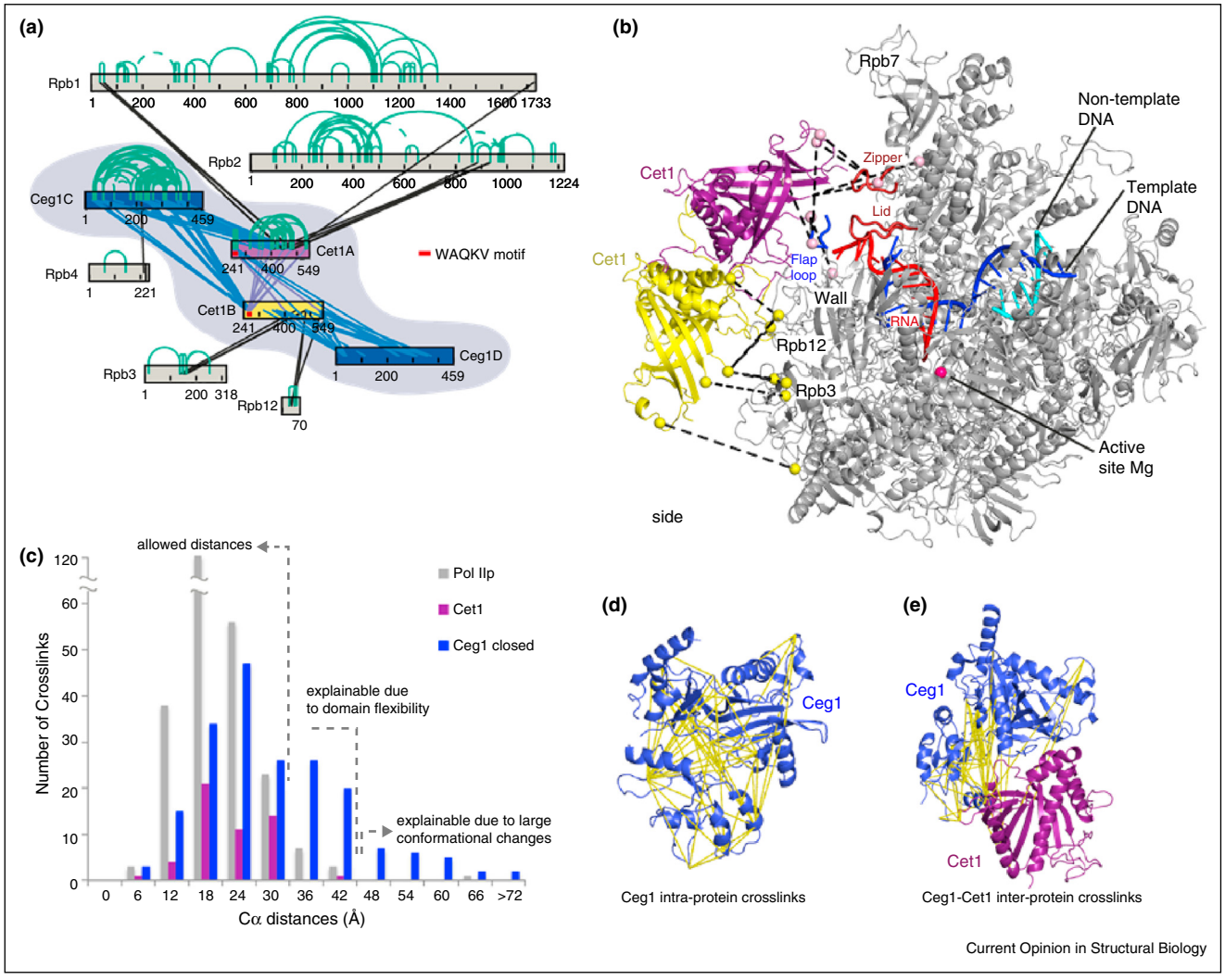
Structural elucidation on large macromolecular assemblies

Although X-ray crystallography and NMR spectroscopy are unsurpassed in the determination of protein structures at atomic level, revealing very large macromolecular assemblies is often refractory to these approaches, mostly because of their large size and their resistance to purification and crystallization. In this regard, XL-MS exhibits its great competence in overcoming these obstacles, and its utility can be remarkably further enhanced through the combination with structural information obtained by other means. In the last couple of years, in particular hybrid approaches that integrate structural data derived from XL-MS, cryo-electron microscopy (cryo-EM), X-ray crystallography, native MS and computational modeling/docking, have led to great achievements in the structural analysis of large macromolecular protein assemblies. In this framework, XL-MS can provide distance constraints to guide computational modeling as well as connectivity maps to determine the general subunit topology. Prominent examples of protein assemblies studied by such hybrid approaches are the TriC/CCT chaperonin system [14•,15,19], the 26S proteasome [13,16•,47], the mammalian mitochondrial ribosome [48,49], the 40S˙eIF1˙eIF3 translation initiation complex [20], the nuclear pore complex [21,23,24•], CRISPR-Cas complexes [18,50], the Pol II-mediator initiation complex [22], and the transcription-coupled pre-mRNA capping complex [51•].

A very recent study showcasing the power of integrated structural biology approaches elucidated the structure and function of the transcription-coupled pre-mRNA capping complex by combining cryo-EM, native MS and cross-linking MS [51•]. The capping enzyme (CE) functionally couples to the transcription by RNA polymerase (Pol) II to carry out its function in pre-mRNA processing. For this study the transcribing Pol IIp-CE complex was reconstituted by binding recombinant CE to yeast Pol II at substoichiometric levels. For the Pol II-CE complex, (1) the stoichiometry of the 720 kDa phosphorylated Pol II complex associated to the hetero-tetrameric CE (Ceg1-Cet1-Cet1-Ceg1) was determined by native MS, (2) the cryo-EM structure was determined at a resolution of 17.4 Å and (3) a total of 527 lysine-lysine

cross-links representing 337 distance restraints were obtained by XL-MS (Figure 2a). Analysis of the inter-protein cross-links between Cet1 and Pol II guided the placement of the Cet1 dimer crystal structure into the EM density (Figure 2b). Furthermore, in contrast to the defined position of the Cet1 homo-dimer, EM and XL-MS data indicated a high degree of variability for Ceg1. As shown in Figure 2c, all cross-links within Pol IIp and Cet1 are either within the cross-linker distance constraints or can be explained by known domain flexibility. However, only a part of the intra-protein cross-links observed within Ceg1 fall into the distance constraints when mapping to the Ceg1 crystal structures. About 60% of the detected cross-links could be explained by the open Ceg1 conformation and 85% cross-links could be explained by the closed Ceg1 conformation. Still many more cross-links (25 cross-links) spanned intolerable distances larger than 40 Å in the known Ceg1 structures indicating that Ceg1 likely remains intrinsically flexible within this complex. Thus, this study underscores that

Figure 2



Cross-linking mass spectrometry defines the architecture of the transcribing Pol IIp-CE complex. **(a)** Cross-link map of the transcribing Pol IIp-CE complex. Cross-links between Pol II subunits were excluded for clarity. Two copies of Cet1 and Ceg1 are shown. Intra-protein cross-links are in green, inter-protein cross-links between Pol II and CE in black, inter-protein cross-links between Cet1 and Ceg1 in blue, and cross-links between Cet1 molecules in lilac. **(b)** Inter-protein cross-links between Pol II and CE (black dotted lines) guided placement of one Cet1 subunit (yellow) near Pol II subunits Rpb3 and Rpb12, and the other Cet1 subunit (purple) at the end of the RNA exit tunnel. **(c)** Cα distance distribution for observed lysine-lysine cross-links. Cross-links spanning distances of 31–40 Å are explained by the conformational flexibility of mobile protein loops. Distance restraints indicate that Ceg1 resides predominantly in the closed conformation, but alternative states apparently coexist. **(d)** Intra-protein cross-links on the *S. cerevisiae* Ceg1 structure (PDB code: 3KYH) in the closed state after domain alignment onto the viral enzyme structure (PDB code: 1CKM). **(e)** Inter-protein Cet1-Ceg1 cross-links between the *S. cerevisiae* Ceg1 structure in the closed state as in panel D. *Source*: Figure and legend are adopted from [51*] with permission.

XL-MS is also a powerful approach to investigate the flexibility of proteins/protein complexes, which is mostly elusive to more static approaches such as X-ray crystallography.

XL-MS of protein assemblies purified by affinity purification

The aforementioned hybrid approaches represent very attractive means for the structural analysis of highly purified large macromolecular protein assemblies. However, obtaining a satisfactory amount of material with the required purity, is still one of the major challenges. Owing to the extensive fractionation approaches and the high sensitivity of the mass spectrometric detection, sample amount and purity are not essential requirements for XL-MS experiments, making this approach also very suitable for studying protein interactions directly in their cellular context. In this way, native protein structures and biologically relevant interactions are more likely to be retained in the experiment, especially since XL-MS is able to capture weak and transient interactions when the cross-linking reaction is performed in cellular lysates or even intact cells.

To provide this kind of information, approaches integrating cross-linking with affinity purification/mass spectrometry (AP-MS) have recently been explored. Although AP-MS contributed significantly to the understanding of protein interaction network and dynamics [52], it renders little structural information on where and how these proteins interact. The combination with chemical cross-linking is able to simultaneously provide the structure and interaction of endogenous mixtures of protein complexes concomitantly present in the cell. In this procedure, cross-linking can be applied either before (*in vivo* cross-linking) or after (on-beads cross-linking) affinity purification. The former one reflects more physiological conditions, but is technically more challenging due to the complex and compact nature of the cell and the need of cell-permeable reagents. The latter one starts with less complex protein mixtures, but may be hampered by the loss of weak binding partners.

An illustrative example is provided by a study of the protein phosphatase 2A (PP2A) network [25••]. In this study, 14 known proteins that were previously detected in the PP2A network were affinity tagged to determine proteins co-purifying with the trimeric PP2A holoenzymes. In each affinity purification experiment, the associated protein complexes were on-bead cross-linked and subsequently analyzed by mass spectrometry. Overall, 176 inter-protein and 570 intra-protein cross-links were identified that involve the interaction between specific trimeric PP2A complexes and many adaptor proteins. The presented work also elucidated the binding interface between the immunoglobulin binding protein 1 (IGBP1) and the PP2A and revealed the topology of the TCP1 ring complex (TRiC) chaperonin interacting with the PP2A regulatory subunit 2ABG using computational docking. Another explanatory example is a cross-linking study of cyanobacteria photosystem complexes [27]. In cyanobacteria, phycobilisomes (PBSs) absorb light and transfer its energy to chlorophylls in photosystem II (PSII) and photosystem I (PSI). To address how these three protein assemblies structurally interact, XL-MS was applied to the cells and the resulting cross-linked PBS-PSII-PSI megaDa complex was isolated by affinity purification of a polyhistindine tag on the C terminus of PSII subunit O (PsbO). Based on the cross-linking results and computational modeling, the organization of the very large PBS–PSII–PSI complex could be successfully characterized, providing a solid basis for understanding how PBSs transfer excitation energy to reaction centers and how the energy balance of the two photosystems is achieved.

Proteome-wide XL-MS studies

Next to AP-based approaches, attempts have also been made to study *in vivo* protein interactions directly by applying proteome-wide cross-linking in a cellular context (e.g. in cellular lysates or even intact cells), allowing the high-throughput investigation of protein structures and interactions. In this case, XL-MS captures information on all proteins in the mixture at the same time, which, in theory, enables simultaneous monitoring of all stable and transient protein assemblies that are susceptible to cross-linking. Considerable amount of efforts have been directed towards this ambitious aim. Examples include proteome-wide cross-linking studies on several different species, such as *E. coli* [29••,30••,53], *C. elegans* [30••], *S. oneidensis* [54], *P. aeruginosa* [55] and *H. sapiens* [32,34,46••] using either conventional cross-linkers [29••,30••] or MS-cleavable cross-linkers [32,34,46••,53–55].

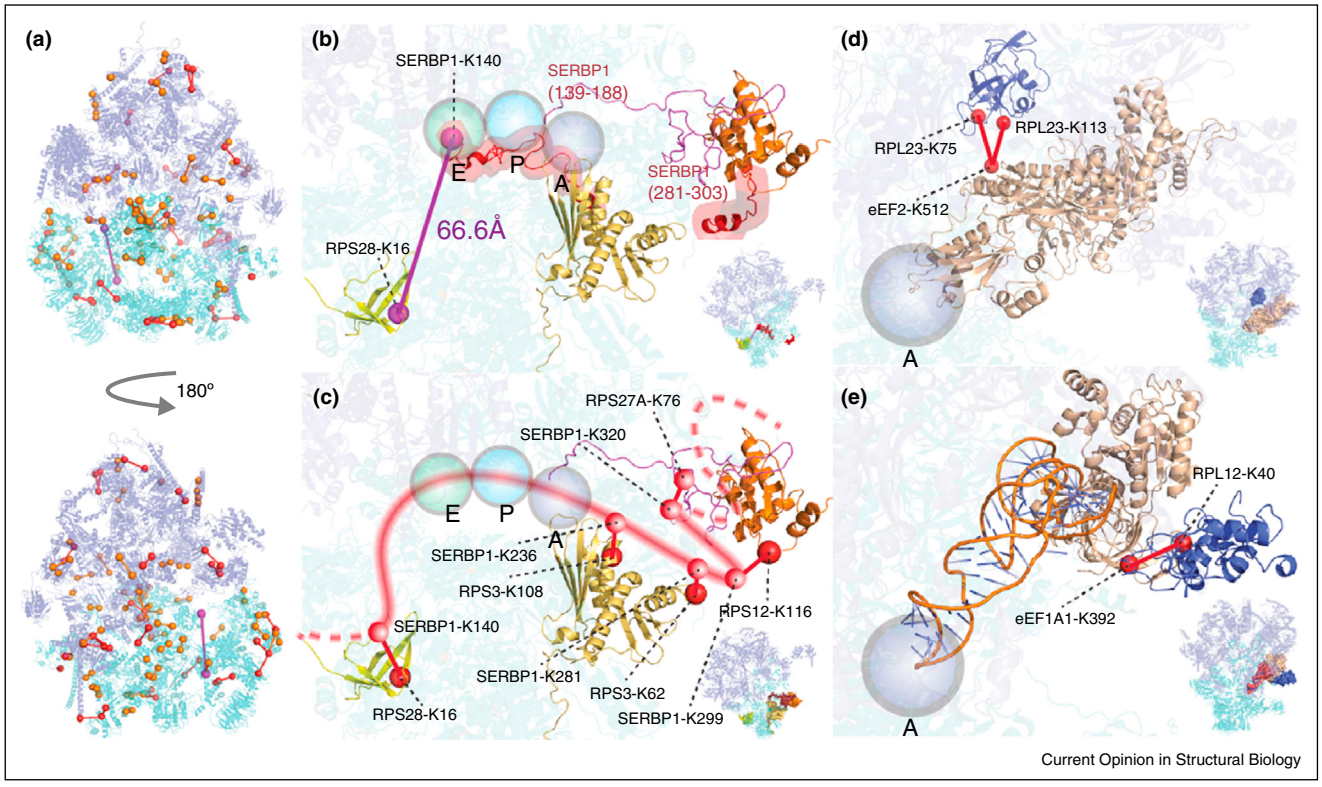
To this end, we refer to very recent work from our lab as an illustrative example. In this study, we described a substantial leap forward in XL-MS demonstrating a three-pronged approach consisting MS-cleavable cross-linkers, sequential collision-induced dissociation (CID) MS^2 and electron-transfer dissociation (ETD) MS^2 acquisitions, and a dedicated search engine XlinkX to rapidly and robustly identify cross-links from a complete human proteome database. We performed XL-MS studies on whole human cellular lysates and confidently identified 2179 unique Lys-Lys cross-links. The confidence of our crosslinking dataset was double validated through a target-decoy strategy and through mapping the observed crosslink distances onto existing high-resolution structures.

Of biological significance, our data firstly provided new structural insights on many protein assemblies. One example is the structural details of the interaction between the 80S ribosomal core complex and plasminogen

activator inhibitor 1 RNA-binding protein (SERBP1) — an mRNA binding protein that has an extremely flexible and extended structure. This discovery was led by the observation of an extremely long cross-link (Cα-Cα distance of 66.6 Å) between SERBP1 and ribosomal protein small subunit 28 (RPS28), while the remaining 78 cross-links that could be mapped on the structural available regions of the human 80S ribosome (PDB 4V6X) are either within the cross-link distance constraints or can be well-explained by in-solution structural flexibility (Figure 3a). This anomalous cross-link (SERBP1-K140-RPS28-K16) suggests that different from the previously proposed SERBP1 localization on the 80S ribosomal core complex, the N-terminus of SERBP1 could alternatively be placed much close to RPS28. Additionally, based on prior knowledge that SERBP1 passes by the P-site, A-site and E-site tRNA binding sites and the five newly identified cross-links between SERBP1 and core ribosomal proteins from our study, we proposed an alternative localization of SERBP1 on the 80S ribosome (Figure 3b,c).

Another advantage of the cross-linking studies performed on whole lysates, compared to purified samples, is the capability of cross-linking in capturing dynamic protein–protein interactions. In our whole human cell lysate cross-linking dataset, we also identified several cross-links between ribosomal core proteins and translation elongation factors (including eukaryotic elongation factor 1 alpha 1 (eEFA1) and eukaryotic elongation factor 2 (eEF2)), which dynamically bind to and release from the ribosome core complex during each translation elongation cycle.

Figure 3



Mapping detected cross-links in whole human lysate on the 80S ribosome. **(a)** All identified cross-links involving ribosomal proteins are mapped onto the available part of the human 80S cryo-EM structure (PDB: 4V6X). Small subunit (40S) is shown in cyan and large subunit (60S) is shown in blue. Cross-links within the DSSO maximum distance constraint of 28.4 Å are indicated by orange (intra-peptide cross-links) and red (inter-peptide cross-links) lines. Cross-links exceeding the maximum distance constraints are represented as magenta lines. **(b)** A zoomed-in view of the anomalous cross-link between SERBP1-K140 and RPS28-K16. SERBP1 (red), RPS28 (yellow), RPS3 (yellow-orange), RPS12 (orange), RPS27A (magenta) are highlighted. The three circles (colored as green, blue and purple from left to right) indicate E-P- and A-tRNA binding sites. **(c)** A schematic positioning of SERBP1 (shown as red line) on the 80S cryo-EM structure based on our cross-linking data. The protein color scheme is the same as in panel B. **(d)** Cross-link mapping of eEF2-K512 with RPL23-K75 and eEF2-K512 with RPL23-K113 onto the human 80S cryo-EM structure (PDB: 4V6X). RPL23 is shown in blue and eEF2 is shown in light brown. **(e)** Cross-link mapping of eEF1A-K392 with RPL12-K40 onto the rabbit 80S cryo-EM structure (PDB: 4UJE). Of note, RPL12 is not included in the rabbit 80S structure therefore human RPL12 was manually positioned onto the rabbit 80S ribosome based on a structural alignment using Pymol (version 1.5.0.4). RPL12 and eEF1A1 are presented in blue and light brown respectively. The tRNA is shown in orange. All high-resolution structures are viewed in Pymol (version 1.5.0.4).
Source: Figure and legend are adopted from [46••] with permission.

We further mapped the identified cross-links onto the cryo-EM structures of the human 80S-eEF2 complex (PDB: 4V6X) and the rabbit 80S-eEF1A-Val-tRNA-GMPPNP complex (PDB: 4UJE) and evidently showed our data were in good agreement with these published structures (Figure 3d,e).

However, due to the large sample complexity and the low abundance of cross-linked peptides, global XL-MS experiments on proteome-wide samples are still very challenging. Further improvements may be applied at both the protein and the peptide level, such as to substantially fractionate protein assemblies by size-exclusion chromatography and/or to more efficiently enrich inter-peptide cross-links from the peptide digestion mixture. For the latter one, affinity labeled cross-linkers, such as protein interaction reporter (PIR) [32] and the azide-A-DSBSO cross-linkers [34] have been developed. In both examples, the cross-linkers are membrane-permeable, enrichable, and MS-cleavable. The enrichment handle on the cross-linker allows the selective isolation of cross-linked peptides from complex mixtures while the MS-cleavable sites have proven to be effective in facilitating the unambiguous identification of cross-linked peptides. Much progress in this arena is to be expected in the near future, helped by further improvements in novel cross-linker designs, XL-MS methods and MS instruments.

Conclusions

Here we described some of the recent advances in XL-MS approaches and their applications on (1) large macromolecular assemblies, (2) affinity purified endogenous protein complexes and (3) naturally occurring cellular protein interaction networks. By providing the distance constraints of the two cross-linked residues, inter-protein cross-links can be very useful in revealing the subunit topology of protein complexes whereas intra-protein cross-links are helpful in refining pre-existing structures, performing cross-link directed modeling and providing extra information on protein flexibility in solution. Integration of XL-MS with cryo-EM, X-ray crystallography, and native MS strengthen this approach further thus enabling us to reveal the architecture of very large multi-protein macromolecular assemblies. Emerging proteome-wide XL-MS studies reveal that XL-MS may provide useful information on protein structures and interactions directly from their natural cellular environment. The synthesis of enrichable and MS-cleavable cross-linkers holds promise to facilitate the identification of cross-linked peptides in a proteome-wide context. However, affinity purification or substantial fractionation of the mixture of proteins/protein complexes (e.g. size exclusion or anion exchange chromatography) may still be required due to the large complexity of the protein/protein complex mixtures. To cast a general outlook on the future, despite all these challenges, technological developments are destined to take place, ensuring that XL-MS will make great contributions to our understanding of the cellular interactome.

Conflict of interest statement

No conflicts of interest to be reported.

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