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Crosslinking and Mass Spectrometry: An Integrated Technology to Understand the Structure and Function of Molecular Machines

Alexander Leitner,[1,*] Marco Faini,[1] Florian Stengel,[1,2] and Ruedi Aebersold[1,3,*]

In recent years, chemical crosslinking of protein complexes and the identification of crosslinked residues by mass spectrometry (XL-MS; sometimes abbreviated as CX-MS) has become an important technique bridging mass spectrometry (MS) and structural biology. By now, XL-MS is well established and supported by publicly available resources as a convenient and versatile part of the structural biologist's toolbox. The combination of XL-MS with cryo-electron microscopy (cryo-EM) and/or integrative modeling is particularly promising to study the topology and structure of large protein assemblies. Among the targets studied so far are proteasomes, ribosomes, polymerases, chromatin remodelers, and photosystem complexes. Here we provide an overview of recent advances in XL-MS, the current state of the field, and a cursory outlook on future challenges.

Chemical Crosslinking as a Tool for Structural Biology

Structural biology makes use of many different techniques to elucidate the 3D structures of proteins and protein complexes. While high-resolution structures have traditionally been obtained by X-ray crystallography, cryo-EM is increasingly able to also generate (near) atomic-resolution models. In recent years, techniques and applications of MS have also rapidly progressed. Earlier studies were largely focused on the large-scale identification and quantification of proteins, whereas recent methods also support queries into the composition, stoichiometry, and spatial arrangement of subunits in a complex. These developments have now further progressed toward generating information that contributes, as part of hybrid structural strategies, to the structure elucidation of large molecular assemblies including protein complexes that perform essential processes in the cell. XL-MS is a particularly powerful mass spectrometric technique in this respect, because it provides several layers of information. Identifying protein–protein contacts through XL-MS confirms physical proximity between subunits because the proteins must be close enough in space to be crosslinked. Localizing the side chains that are connected restricts this proximity to certain regions (e.g., domains or even single helices or loops). Finally, the structure of the connected side chains and the crosslinker moiety impart a distance restraint that can be used for molecular modeling purposes because an upper

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Chemical crosslinking followed by the mass spectrometric analysis of cross linked peptides (XL MS) identifies con tact sites between residues within a single or between multiple proteins.

The application of XL MS to many bio logically relevant molecular machines has been shown, with a rapidly growing number of successful studies reported in the past 2 3 years.

Crosslinking data are useful in integra tive modeling workflows by providing distance restraints on the surface of folded proteins and complexes. XL MS has been shown to be particularly powerful in combination with 3D cryo electron microscopy.

[1]Department of Biology, Institute of Molecular Systems Biology, ETH Zürich, 8093 Zürich, Switzerland
[2]Department of Biology, University of Konstanz, 78457 Konstanz, Germany
[3]Faculty of Science, University of Zürich, Zürich, Switzerland

*Correspondence:
leitner@imsb.biol.ethz.ch (A. Leitner) and aebersold@imsb.biol.ethz.ch (R. Aebersold).

bound of the physical distance can be calculated for a particular crosslinker. An advantage of XL-MS over other structural techniques is that it can deal with limited sample heterogeneity or dynamic complexes as it provides an averaged ensemble measure.

The general approach of XL-MS is to chemically crosslink proteins in their native or native-like state then generate crosslinked peptides by enzymatic digestion of the crosslinked samples and identify the sequence of the crosslinked peptides via tandem MS. Most commonly, a purified protein complex is incubated with a crosslinking reagent that forms covalent bonds between reactive surface-exposed amino acid side chains and the samples are digested with trypsin. The resulting peptides can be enriched for crosslinked peptides and are analyzed by liquid chromatography tandem MS (LC-MS/MS). Computational analysis of the MS/MS data enables sequence assignment of the crosslinked peptides as well as the localization of the exact crosslinking sites. An overview of the general workflow and recent innovations are presented in Figure 1 (Key Figure).

Neither chemical crosslinking nor the use of MS for the identification of single crosslinked proteins is by itself a novel concept. However, due to multiple major technical obstacles it had been impossible until recently to directly and reliably identify crosslinked peptides from protein complexes by MS (see [1–4] for recent reviews). After early work on individual proteins [5–7] and protein complexes [8,9], notably by the Sinz and Rappsilber groups, Aebersold and coworkers introduced the first robust general workflow by optimizing wet-lab protocols and the development of the publicly available xQuest/xProphet open-source software suite for the analysis and validation of crosslinks from large protein complexes by MS [10–12].

Over the past few years the field has seen significant progress and several methods to enrich crosslinks [13–15], various crosslinking chemistries [9,10,15–22], and the introduction of multiple detection and identification strategies [9–11,23–28]. Statistical models that differentiate true from false identifications have also been developed [10]. Recent additions to the field also include various tools to visualize crosslinks as networks of connected residues or as spatial restraints on the surface of protein structures, a great improvement over the manual annotation and mapping of the early days [29–32]. Although space limitations do not allow us to cover all of the exciting advances in the field in this review, we highlight here some particularly noteworthy methodological developments. We then discuss how these advances have provided new insights into the structure and function of complexes such as proteasomes, ribosomes, polymerases, chromatin remodelers, photosystem complexes, and their associated factors.

Recent Advances in XL-MS Methodology

The main bottlenecks in XL-MS have long been the lack of dedicated methods to specifically enrich crosslinked peptides from the complex peptide mixture generated by the digestion of large protein complexes and, even more so, the reliable identification of crosslinked peptides [1]. Over the past few years there has been great progress in making workflows more robust and now multiple relatively mature approaches exist, at least for the analysis of abundant recombinantly expressed or reconstituted protein complexes (see Applications of XL-MS for Elucidation of the Structure and Function of Large Protein Complexes). Numerous applications of XL-MS have shown that the methods are robust but the number of obtained crosslinks is sometimes marginal. This has created growing interest in methods that increase the arsenal of available crosslinking chemistries and thus the number of identified crosslinks. The most commonly used crosslinking reagents are homobifunctional active esters such as disuccinimidyl suberate (DSS) or bis(sulfosuccinimidyl) suberate (BS^3) that induce nucleophilic attacks on primary amines and thus rely on the coupling of lysine residues. One exciting way to expand beyond this established strategy is the recently developed crosslinking chemistry specific for carboxyl groups using homobifunctional dihydrazides as crosslinking reagents [18,33].

Key Figure

An Overview of the Crosslinking Mass Spectrometry (XL-MS) Workflow

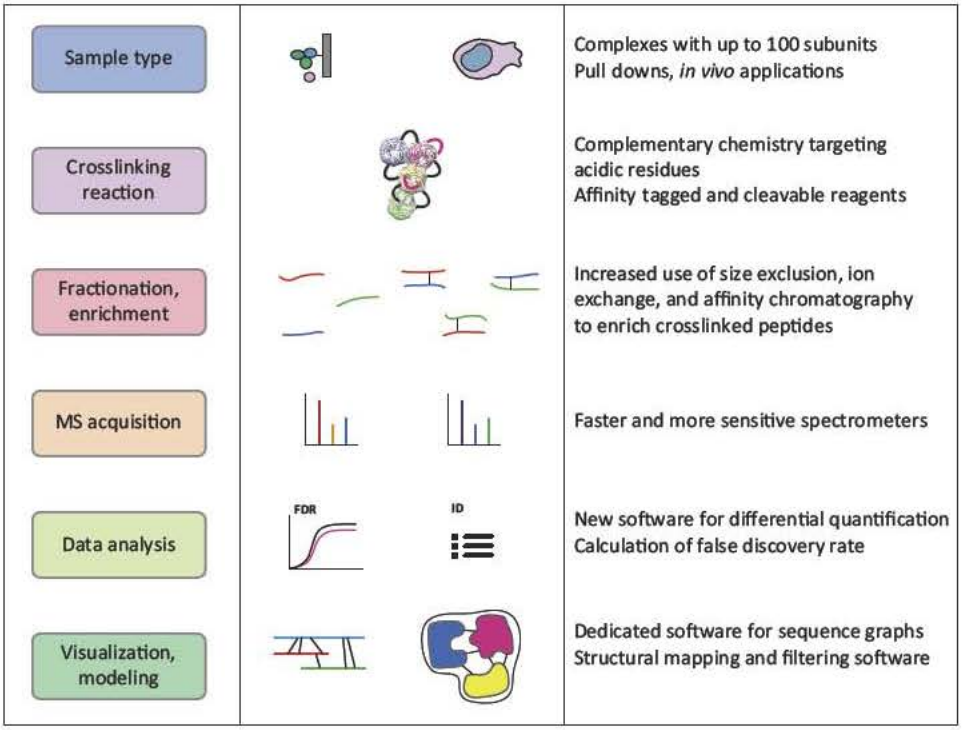

Figure 1. A schematic representation of the crosslinking workflow (left and middle) is accompanied by recent innovations in the field (right). XL MS is generally performed on purified samples. Alternatively, cells can be incubated with crosslinkers (*in vivo* crosslinking). The sample is then digested by a protease into peptides and crosslinked peptides (with black lines) are enriched or fractionated by size exclusion chromatography, ion exchange chromatography, or affinity chromatography. Enriched or fractionated samples are separated by liquid chromatography and analyzed by tandem MS. The crosslinked peptides are identified (ID) and an estimate for the false discovery rate is obtained (FDR). A list of validated identifications is compiled. The validated crosslinks can be displayed as sequence graphs, visualized on atomic structures, or used for assembly modeling.

This crosslinking chemistry can provide distance restraints that are highly complementary to those obtained from lysine crosslinking [19,34,35], thereby considerably expanding the number of crosslinks and thus the amount of structural information that can be obtained from a protein complex. However, carboxyls are inherently less reactive than amine groups and require a more elaborate procedure for crosslinking. Zero-length crosslinking, using carbodiimides to bridge amino and carboxyl groups directly without insertion of a spacer, has recently also been applied to larger protein complexes [36,37], although data analysis in such experiments remains more challenging.

The abovementioned reactions are specific for one or several amino acids, but more promiscuous reagents also exist, including those with photoreactive groups [38,39]. Expanding the list of reactive residues increases the chances that proteins with fewer acidic or basic residues, such as membrane proteins, can also be crosslinked. A completely unspecific chemistry would make

the technique less suited for the analysis of larger complexes. The use of heterobifunctional reagents containing one specific and one photoreactive group is an attractive solution to circumvent such problems [40] and promises high crosslink coverage for individual proteins [41]. A somewhat different application of photochemical crosslinking was used in a recent breakthrough study that introduced the first semiautomated workflow for assigning RNA–protein binding sites [42]. In this case the intrinsic photochemical reactivity of the nucleobases uracil and 5-thiouracil can be exploited. Even if the analysis of such data remains extremely demanding, this study does open new horizons for extending the concept beyond protein–protein interactions.

So far, XL-MS has been primarily used to study the structure of protein complexes as static entities. Because MS provides not only qualitative but also quantitative information, the development of 'quantitative' XL-MS (qXL-MS) workflows is an obvious next step. Addition of a quantitative dimension is likely to enable deeper insight into the compositional and conformational differences of the protein complexes present in different biological states. These changes would be reflected in changes in the abundance of specific crosslinks. The potential of qXL-MS remains underexplored, partly due to the lack of adapted experimental workflows and a lack of software tools that can handle qXL-MS data [43]. Initial studies have used manual data extraction and quantification and were therefore not easily scalable to more ambitious projects [43–45]. However, Schmidt *et al.* have provided an exciting early example of the potential of this method by comparing unphosphorylated and phosphorylated states of a spinach chloroplast ATPase [44]. Recently, the Aebersold group has introduced a new software solution for the automated analysis of qXL-MS datasets [46]. They also demonstrated its use for the structural analysis of protein complexes that exist in distinguishable stable conformational states such as the chaperonin TRiC/CCT.

Despite these recent advances, crosslinking restraints alone are not sufficient to elucidate the precise structure of a protein complex. Thus, it was realized that combining XL-MS with other structural approaches in a hybrid setting offers great advantages (see The Role of XL-MS in Integrative Structural Biology) [47]. For example, it was demonstrated that a more integrated view of the subunit arrangement of an intact protein complex can be obtained by examining a sample with XL-MS, native MS, and ion-mobility spectrometry coupled to MS so that the complementary structural MS methods provide additional and orthogonal restraints compared with using XL-MS alone [48]. Advanced computational pipelines can support integration of the different types of data from crosslinking and from other sources – such as complementary experimental or computational methods – to facilitate the unbiased use of crosslinking and other restraints to generate reliable models for protein complexes [49–52]. This led to the generation of a generic hybrid approach for the accurate modeling of large protein assemblies, where XL-MS together with partial crystal structures of subunits were successfully integrated with modeling. This integrated method generally permits a large number of low-resolution restraints to be effectively integrated and optimized. As a key example of its usefulness, it was used to elucidate the structure of an intact translation initiation factor that had evaded crystallization efforts for two decades, bound to the ribosome [49].

XL-MS is not limited by the size of a protein complex and provides a wealth of information on the connectivity, interaction, and relative orientation of subunits within a complex. A crosslink contact also contains (relatively low resolution) spatial information in itself. Recent technological advances [53,54] have now rendered this method applicable to physiologically important biological assemblies that are difficult to examine using more traditional structural techniques. This technology is therefore optimally suited for studying the architecture of large protein complexes and their interaction partners in their native environment and on a medium to large scale.

The Role of XL-MS in Integrative Structural Biology

Integrative Structural Biology

Common structural techniques such as X-ray crystallography, NMR spectroscopy, and 3D EM have strengths and specific application fields but no technique by itself may perform as well as a combination of methods when complex molecular machines are being analyzed. Therefore, in recent years structural biology has seen the rise of a new methodological paradigm in the form of integrative structural biology and modeling. It was pioneered by creating a model for one of the largest and complex macromolecular machines: the nuclear pore complex [55]. Integrative structural biology determines structure in three stages (Figure 2). First, a comprehensive list of 'parts' of the complex of interest is compiled. These parts can include, for example, atomic models of single subunits in addition to the sequence of the component proteins. Flexible regions or regions for which no structural information is available can be represented by beads (as placeholders) occupying the space of single amino acids, secondary structure elements, or entire protein domains [56]. Additionally, known interactions between parts, such as copurification data derived from affinity-purification MS (AP-MS) and data from yeast two-hybrid experiments can be integrated. Available data from XL-MS or other structural MS techniques, and from NMR spectroscopy or electron paramagnetic resonance, are also translated into spatial restraints. In the second stage, these restraints are combined into a single scoring function. Multiple sampling of such functions generates a set of assembly models that have

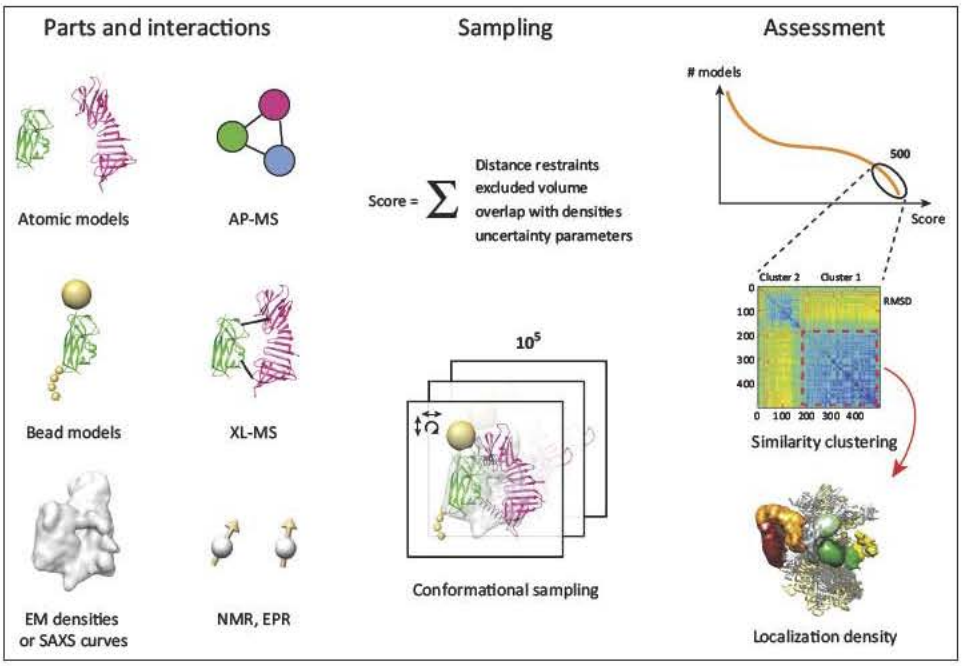

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Figure 2. The Role of Crosslinking Mass Spectrometry (XL MS) in Integrative Structural Biology. Integrative structural biology can be subdivided in three consecutive steps shown from left to right. First, structural components such as atomic models and electron microscopy (EM) density maps are gathered. Parts that are not described at atomic resolution are represented by beads occupying the space of amino acids or protein domains (left). Interaction data between parts are collected from affinity purification MS (AP MS), XL MS, NMR, or electron paramagnetic resonance (EPR) experiments. Interactions are then translated into distance restraints and all restraints are combined into a scoring function. Applying translations and rotations to the individual parts is equivalent to extensively sampling the scoring function. Models with higher scores satisfy more of the restraints (center). Finally, models are ranked by their score and the best models are clustered by their structural similarity [root mean square deviation (RMSD)]. A representative cluster (red broken line square) is then averaged into a localization density map that illustrates the most probable location of the individual parts (right). SAXS, small angle X ray scattering. Adapted from [49].

different degrees of consistency with the input data. In the third stage, models are scored based on how well they satisfy spatial restraints and one can then generate a consensus model [56]. This workflow is currently implemented in generic software such as the Integrative Modeling Platform (IMP) [57], HADDOCK [58,59], and ROSETTA [60]. Thus, by integrating different levels of heterogeneous structural information, integrative structural biology is able to provide structural models of assemblies with large numbers of subunits and some degree of heterogeneity and flexibility.

Restraints from XL-MS

XL-MS plays a pivotal role in integrative structural biology. The restraints derived by crosslinking are particularly important to determine the topology and relative orientation of the individual subunits. XL-MS provides definitive binary interaction data (e.g., subunit A is close in space to subunit B) and spatial restraints between proteins with a resolution of several amino acids at the primary sequence level (limited by the location of crosslinkable residues). These restraints are in the range 7–30 Å, with a median distance of approximately 15 Å for the most commonly used lysine-reactive reagents [14,61,62] and slightly shorter for carboxyl-reactive hydrazides and zero-length crosslinks [18]. This allows the determination of the proximity and, if multiple crosslinks in complementary regions are found, the relative orientation of the subunits. Additionally, the technique can detect interactions of the same site with multiple target sites (one-to-many connections) and thus identify mutually exclusive assemblies. Consequently, crosslinks can provide a strong discriminant between false and true solutions generated by integrative modeling.

Application Examples

XL-MS data provide orthogonal information to low-resolution (10–20 Å) 3D EM density maps. For example, the Chait group proposed an integrative model for the nuclear pore subcomplex Nup84 combining data from X-ray crystallography, EM, and XL-MS [50]. With the goal of obtaining a large set of crosslinks, they combined the widely used reagent DSS, which targets lysine residues, with a carbodiimide to generate zero-length crosslinks. By combining validated crosslinks from the two chemistries they obtained a set of 59 and 47 intermolecular crosslinks, respectively. The resulting model is consistent with the recently published crystal structure [63]. A combination of two flavors of 3D EM – cryo-electron tomography and single-particle EM – and XL-MS was employed by the Beck group to define restraints within the purified Nup87 complex and between different copies of the same complex within the assembled nuclear pore complex [64].

Another example of integrative structure modeling applied to a large macromolecular machine is the molecular structure of eukaryotic initiation factor (eIF) 3 [49]. Here the authors combined X-ray crystallography, single-particle EM, and XL-MS. A large set of experiments allowed comprehensive mapping of the lysine–lysine interprotein interactions between the eIF3 complex and the 40S ribosomal subunit (155 crosslinks) and within the 40S ribosomal proteins (461 crosslinks). Given the size of the dataset, the recurrence of a particular crosslink could be used as a further confidence metric to assess the validity of the created structural model [49].

Limitations

The major limitation of XL-MS from the structural point of view is that it cannot directly determine the relative stoichiometry of subunits in a complex, although this information can be derived from complementary quantitative MS methods. XL-MS also cannot easily distinguish between intra-subunit crosslinks and crosslinks between members of a homomeric interaction. Homodimeric complexes can be reconstituted from mixtures of ^{14}N- and ^{15}N-labeled subunits [65,66] but this approach is not easily extensible to larger complexes. For the same reason, interactions of different copies of the same subunit with different partners cannot yet be deconvoluted; that is, attributed to single subunits.

Applications of XL-MS for Elucidation of the Structure and Function of Large Protein Complexes

Whereas the first applications of XL-MS in the early 2000s focused on individual proteins or small complexes, the Rappsilber group demonstrated its use for larger protein assemblies in seminal studies on the tetrameric kinetochore subcomplex NDC80 [8] and RNA polymerase II (Pol II) in complex with transcription initiation factor IIF (TFIIF) [9], a 15-subunit, 670-kDa complex. In the latter study, more than 90 high-confidence lysine–lysine contacts between Pol II and TFIIF were used to position the initiation factor on the known structure of the polymerase (Figure 3A). In the following years, more laboratories specializing in MS and proteomics adopted the technique, although it took time for the technique to become accepted in the structural biology community. The more general acceptance resulted from improvements in wet-lab protocols, the availability of more sensitive MS instrumentation, and more robust software that was able to routinely and robustly deal with larger datasets, as outlined above.

In recent years, the methodology has been applied to several molecular machines involved in the most essential cellular processes. We discuss selected examples in the following paragraphs. Box 1 discusses the emerging expansion of the XL-MS concept to the proteome scale.

Proteasomes

The proteasome is the most important cellular machinery for protein degradation. It comprises two main parts, the 19S cap particle and the 20S core particle, which constitute the 26S holocomplex. Although the structure of the 20S core particle was determined by crystallography two decades ago [67], the structure of the complete 26S assembly has remained elusive as the whole complex remained refractory to crystallography. Instead, new insights into the architecture of the proteasome were gained by XL-MS. The first application of XL-MS to the proteasome dates back to 2006, when Robinson and coworkers used native MS and crosslinking to obtain partial interactions between subunits in the 19S subcomplex of the *Saccharomyces cerevisiae* proteasome [68]. However, the actual crosslinking sites were not determined in this case. Spatial restraints obtained from XL-MS were used to eventually characterize the complete subunit architecture of the 26S proteasome [69,70]. The architecture of the intact proteasome derived from a hybrid method with contributions from XL-MS, EM and other methods proved to be nearly identical to the architecture obtained from an independent cryo-EM project that was published at the same time [71]. In addition, the proteasome has served as a model complex for the development of new experimental and computational workflows connected to XL-MS [11,14,18,72].

Ribosomes and Associated Proteins

Because of their essential and diverse function, ribosomes are among the most frequently studied targets in structural biology of large protein complexes, including structural proteomics and XL-MS. Lauber and Reilly demonstrated the utility of a new lysine-reactive crosslinking reagent, diethyl suberthioimidate, by applying it to the *Escherichia coli* ribosome [73]. Later, the same group used this method to localize the binding site of ribosomal protein S1 on the complex [74]. Due to its dynamics, X-ray crystallography studies cannot observe S1, a situation that highlights the relevance of XL-MS as an alternative approach for probing subunit relationships in flexible assemblies. The ribosome is dependent on interactions with other proteins and protein complexes for its biogenesis and function. For example, association with eIF3 is required for translation initiation in eukaryotes. Spatial restraints from XL-MS were used in combination with EM, X-ray crystallography of selected subunits, and integrative modeling to elucidate the architecture of the 40S ribosome–eIF1–eIF3 complex of *S. cerevisiae* [49] (Figure 3B). In this study, 965 intra- and interprotein crosslinks were obtained on the complete assembly, making it the most comprehensive XL-MS data set from individual complexes to date. Additional crosslinking studies on eIFs were performed by the Robinson group [75,76].

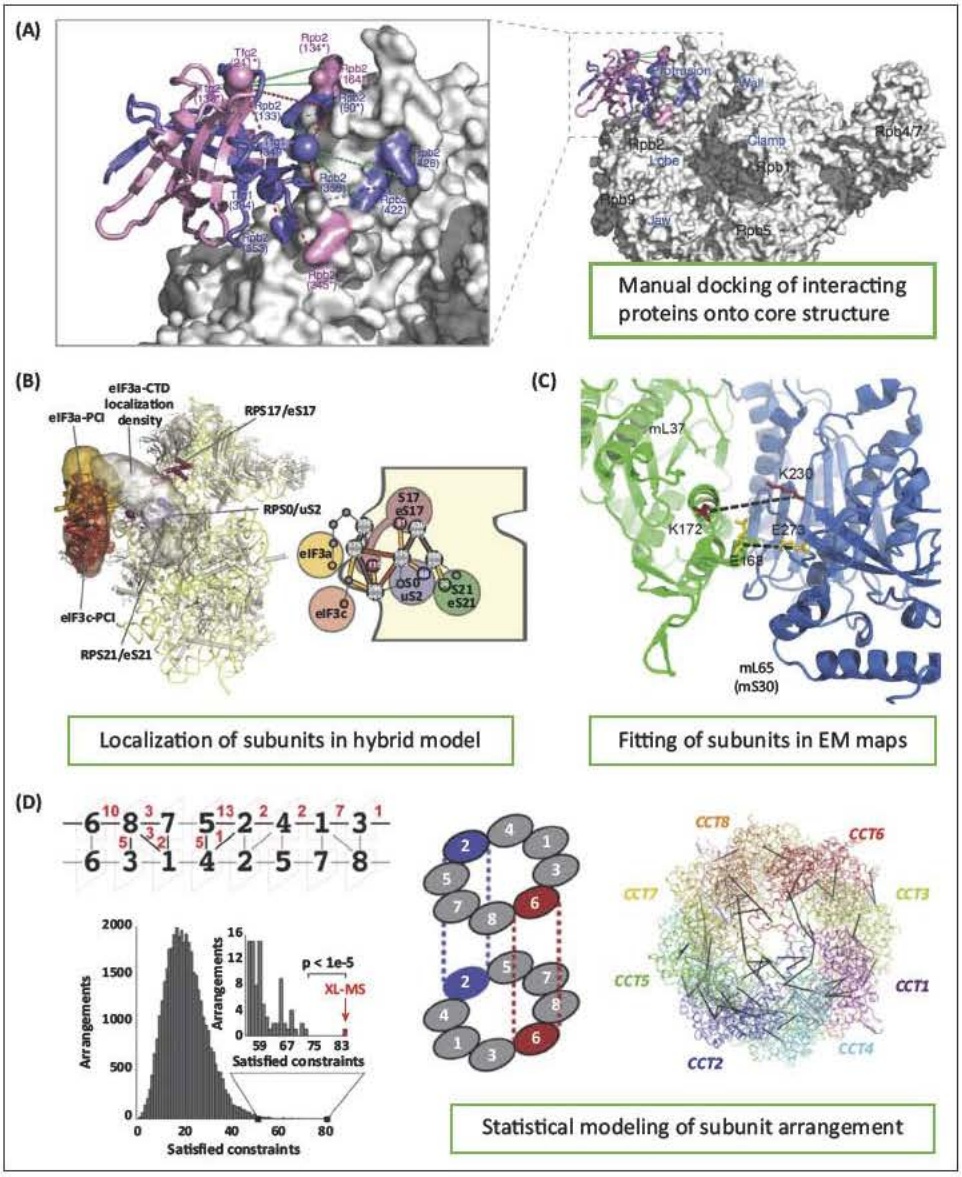

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Figure 3. Examples of Various Uses of Crosslinking Restraints for Elucidation of the Structure of Protein Complexes. (A) Manual positioning of the transcription initiation factor IIF (TFIIF) dimerization domain onto the structure of RNA polymerase II. Reproduced, with permission, from [9]. (B) Localization of the unordered C terminal domain of eukaryotic initiation factor 3a (eIF3a) on the 40S ribosome by crosslinking guided hybrid modeling. Reproduced from [49]. (C) Positioning of subunits of the mitochondrial ribosome on cryo electron microscopy (cryo EM) density maps. Broken lines connect crosslinked residues. Reproduced from [34]. (D) Statistical derivation of the subunit arrangement of the TCP 1 ring complex (TRiC)/chaperonin containing TCP 1 (CCT) chaperonin. CCT subunits are numbered from 1 to 8. Solid lines indicate crosslinked subunits; broken lines indicate which pairs of homotypic inter ring contacts occur. Reproduced, with permission, from [97].

The mitochondrial ribosome (mitoribosome) differs substantially from the cytosolic form of the complex in eukaryotes in terms of both structure and cellular function. Specifically, its RNA content is only about one-third of the total mass, compared with two-thirds for the cytosolic ribosome, and it is responsible for the synthesis of only a limited number of hydrophobic

Box 1. Crosslinking of Protein Networks and Whole Proteomes

Recently, the first studies to apply XL MS to a larger set of complexes that were affinity purified directly from cells have emerged [48,110]. Herzog *et al.* studied the interaction network of human protein phosphatase 2A (PP2A) [110] by purifying proteins associating with a total of 14 different bait proteins (PP2A subunits and known interactors) and supported the resulting network with XL MS derived spatial restraints obtained from crosslinking directly on the affinity beads. The Aebersold and Robinson group studied the assembly of the proteasome lid in yeast using, among other methods, XL MS on pull downs from tagged proteasome subunits [48]. The extension of crosslinking to such complexes expressed at endogenous levels will make the concept more accessible to study protein interactions in unprecedented detail. Current limitations of this approach are the limited amount of material that can be recovered by affinity purification and the low throughput for larger numbers of baits.

The extension of XL MS to the proteome scale has made the probing of protein interactions directly in living cells or in cell lysates possible to some extent. Using the protein interaction reporter concept based on gas phase cleavable, affinity tagged crosslinking reagents, Bruce and coworkers have shown applications to a diverse range of organisms such as *Shewanella oneidensis* [111], *Escherichia coli* [112 114], *Pseudomonas aeruginosa* [115], and even human cell lines [116]; other groups have reported similar concepts [117 119]. Recently, the group of Bruce also demonstrated that a quantitative dimension can be added to proteome wide crosslinking data with the help of metabolic stable isotope labeling [120]. These potentially exciting approaches still have difficulty in identifying larger numbers of interactions between proteins or protein complexes that are not highly abundant like the ribosome, certain chaperones, or histones, but improvements in experimental protocols and instrumentation will increase the coverage in the future, as exemplified by recent work from Heck and coworkers [121].

membrane proteins. Unfortunately, its low abundance has made the mitoribosome practically inaccessible to structure elucidation by crystallography. Thus, high-resolution cryo-EM was used in several recent projects to decipher the architecture of the mitoribosome of yeast and mammals [34,35,77–80]. For three of these studies [34,35,77], XL-MS contributed restraints that were critical in localizing individual subunits within this massive complex (Figure 3C).

Polymerases and Associated Complexes

RNA transcription is an intricate process that involves the interplay of several different protein complexes, among them RNA polymerases themselves and the Mediator complex that acts as a transcriptional activator, as well as various transcription factors that assemble with RNA Pol II in the preinitiation complex (PIC). As mentioned above, the Pol II–TFIIF complex was the first large assembly studied in detail by XL-MS [9]. In recent years, XL-MS has provided important insights into the architecture of many of these complexes. This includes information about the subunit organization of the polymerases themselves as well as the organization of polymerases with additional proteins into higher-order functional units. Studies have targeted RNA Pol I [81,82], II [83], and III [84] as well as the Pol II–PIC complex [85,86] and the Pol II-capping enzyme complex [87]. A subcomplex of the Mediator complex (the so-called middle module) [88] and the Mediator head module in complex with the C-terminal domain of Pol II [89] were also investigated by XL-MS. More recently, XL-MS was used to provide spatial restraints on the Mediator core and the Pol II–Mediator core initiation complex in the most comprehensive study on polymerases and associated complexes to date [90].

Chromatin Remodelers

Chromatin remodeling complexes are responsible for the reorganization of nucleosomes through various mechanisms. Recent crosslinking studies have targeted several families of remodeler enzymes. For example, the Aebersold group contributed to two hybrid structural biology projects focusing on the remodelers INO80 [91] and Swi2/Snf2-related 1 (SWR1) [92]. In both cases, XL-MS data aided in positioning individual subunits into EM maps. For INO80, crosslinks between the remodeler and the nucleosome were obtained, providing a first glimpse into the regulatory activity of the complex. Recently, Vermeulen and coworkers used AP-MS to examine the interaction network of the nucleosome remodeling and deacetylase complex (NuRD) remodeler and used XL-MS to study the architecture of the human NuRD [93].

Complexes Involved in Photosynthesis

Photosynthesis in organisms such as cyanobacteria and red algae involves light absorption and energy transfer via protein complexes. Several recent studies have taken advantage of XL-MS to obtain information about the spatial proximity of individual proteins in these molecular machines. For example, Liu *et al.* studied a phycobilisome–photosystem I–photosystem II 'megacomplex' in the cyanobacterium *Synechocystis* [94]. The architecture of phycobilisomes from *Thermosynechococcus vulcanus* was also studied by crosslinking [95]. Finally, the complex between the Fenna–Matthews–Olson antenna protein and the reaction center core complex in *Chlorobaculum tepidum* was also probed by XL-MS [96].

Other Complexes

In addition to the families of molecular machines discussed above, several other large protein complexes that have constituted longstanding problems in structural and cell biology have already been studied by XL-MS. Some notable examples include: the group II chaperonin TCP-1 ring complex (TRiC)/chaperonin containing TCP-1 (CCT) [97–99], including the use of XL-MS to identify its substrate-binding sites [99] (Figure 2D); the SAGA transcription coactivator complex [100,101]; polycomb repressive complex 2 [102]; spinach F-type ATPase [44]; the *Pyrococcus furiosus* Cmr complex, part of the CRISPR system in prokaryotes [20]; and the metabolon, an assembly of mitochondrial enzyme supercomplexes [103].

Very large macromolecular assemblies that may comprise hundreds of individual proteins currently remain inaccessible to a comprehensive XL-MS analysis in their entirety. This is partly because of the difficulties in isolating sufficient amounts of them as homogeneous entities, but also because experimental challenges with the currently available instrumentation do not allow comprehensive coverage. However, such very large machines can be targeted by studying partial assemblies with the intent to then computationally assemble the subcomplexes into the structure of the whole system. For example, several subcomplexes of the nuclear pore complex have been studied using crosslinking strategies [50,64,104,105]. Similarly, complexes associated with the kinetochore have been studied by XL-MS [8,106–109].

In summary, the application of XL-MS to this diverse group of molecular machines has provided crucial information about their hierarchical organization and has supported complementary experimental and computational structural techniques. XL-MS, therefore, has been firmly established as an important component of the hybrid structural biology toolbox.

Concluding Remarks

XL-MS has made essential contributions to structural biology that are reflected in the significant increase in the number of published applications of this technology. It is encouraging to see increasing interest in and a broad range of successful applications of a method that only a few years ago was restricted to proof-of-principle experiments on model proteins. It can be expected that the more widespread acceptance of XL-MS as a viable part of structural biology projects will result in exciting new directions for this technique. The immediate challenges for XL-MS are related to two different parts of the workflow (see Outstanding Questions). The field remains heterogeneous, with various experimental protocols and software applications, and this variety may seem intimidating to newcomers. It can be expected that in the coming years preferred workflows will emerge, and this could be accelerated by community efforts related to standardization and benchmarking. Samples of ever-increasing complexity will be studied by XL-MS; however, whether the eventual goal of comprehensive interaction profiling of whole proteomes is achievable remains to be seen. Nevertheless, XL-MS studies of complexes that are partially purified from their native environment will certainly provide new insights about protein interaction networks and their changes on perturbation; for example, as a result of mutations connected to diseases. If such assemblies can be probed routinely at considerable depth and from limited

Outstanding Questions

How can we further increase the amount of information from XL MS experiments for purified complexes?

How can we better integrate XL MS data in modeling pipelines?

How can we expand the concept of XL MS to characterize cellular com plexes in their near native state at the same depth of coverage achieved for purified complexes?

How can we make this technology more accessible to non experts?

sample amounts, this will solidify the relevance of crosslinking-based methods not only in structural biology but also in systems biology, thus advancing the convergence of structural and cell biology.

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