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Editorial

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Chemical Cross-linking Mass Spectrometry for Profiling Protein Structures and Protein-Protein Interactions

Xiaobin Xu†*

Center for Biomedical Mass Spectrometry, Boston University School of Medicine, Boston, MA 02118, USA

Introduction

Profiling protein structures and protein-protein interactions is crucial to define the protein functions in biological systems. With the advancement in mass spectrometry (MS), many MS based methods have been developed to elucidate protein structures and protein-protein interactions, including native MS [1], protein foot printing [2,3], hydrogen-deuterium exchange mass spectrometry (HDX-MS) [4], and chemical cross-linking mass spectrometry (XL-MS), providing great alternatives to the conventional methods, like X-ray crystallography, NMR spectroscopy, co-immunoprecipitation, protein microarrays, yeast two-hybrid, and affinity purification-mass spectrometry. XL-MS have been successfully used to investigate protein structures, protein assembles, and protein-interaction networks [5,6].

Protein cross-linking is a process of formation of covalent bonds that links one protein to another. Chemical cross-linkers react with proteins forming three types of products: inter-molecular cross-linking, intra-molecular loop-linking, and mono-linking. All three products provide useful information about protein structures and protein interactions. Inter-molecular cross-linking is formed by a cross-linker bridging between two polypeptide chains. Inter-molecular cross-linking can be used to identify protein interacting partners as well as interaction sites. Intra-molecular loop-linking is generated by a cross-linker connecting two reactive residues on the same polypeptide chain. Intra-molecular loop-linking gives insight into protein topologies and structural information based on the distance proximity provided by the known spacer length of the cross-linker. Mono-linking is created by one reactive end of the cross-linker attaching to the protein while the other reactive end hydrolyzing to form a free-arm. Mono-linking reveals solvent accessibility of amino acid residues since the residues on protein surfaces are more likely to react with cross-linkers.

Two approaches can be applied to study protein cross-linking. A typical "bottom-up" protein cross-linking experiment contains the following steps: 1) Incubation of protein complexes with cross-linking reagents to allow covalently stabilize transient interactions within the range of the cross-linking reagents, 2) Digestion of the cross-linked proteins with proteases, 3) Enrichment of the cross-linked peptides by physio-chemical properties, 4) Detection of the enriched cross-linked peptides using LC-MS/MS, 5) Data analysis to identify cross-linked peptides to reveal protein interaction partners as well as the interaction sites. Alternatively, protein cross-linking can be conducted using "top-down" approach [7], in which the cross-linked intact proteins are directly injected into a mass spectrometer without prior proteolytic digestion and various MS/MS methods are used to dissociate proteins to localize cross-linking sites. Although the "top-down" approach provides direct analysis by eliminating time consuming steps in the "bottom-up" approach, it suffers from restrictions on sample purities and protein sizes.

Strengths and Challenges of Chemical Cross-linking Mass Spectrometry

Chemical cross-linking mass spectrometry provides a number of strengths. First and foremost, cross-linking can take a snap-shot of real-time dynamic interaction networks on the scale of the entire interactome *in vitro* and *in vivo* [8-10]. Also, cross-linking can not only identify the direct interacting partners but also determine the interaction sites at the same time. Moreover, cross-linking can catch and stabilize transient or weak interactions. Additionally, cross-linking experiments require less material and have less stringency on sample purity as compared to many other methods.

Although chemical cross-linking in combination with mass spectrometry has been a powerful tool to study protein-protein interactions, there are several challenges associated with this approach. First of all, a list of cross-linkers with various chemoselectivities and lengths needs to be evaluated in order to determine the suitable cross-linkers for the protein complexes under investigation. In addition, the abundance of cross-linked peptides is significantly lower than that of unmodified peptides and mono-linked peptides, especially in complex biological systems. To address this challenge, effects have been made to enrich cross-linked species and to design innovated cross-linking reagents that can enhance the detection of cross-linked peptides. Furthermore, computational algorithms used identify and validate the cross-linked peptides and cross-linked sites require improvements, especially for processing complex protein mixture cross-linking. Many novel cross-linking reagents and bioinformatics tools have recently been developed to address these challenges, as discussed below.

Cross-linking Reagents

Common chemical cross-linkers are non-cleavable homobifunctional and heterobifunctional cross-linkers. Homobifunctional cross-linking reagents contain identical reactive groups at both ends, connecting with a carbon-chain spacer at a defined distance. The heterobifunctional cross-linking reagents contain two different reactive groups that target different functional groups on proteins. The most common reactive group is N-hydroxysuccinimide (NHS), which is targeted to primary amines in the side chains of lysine residues and protein N-termini. Another common used reactive group is maleimide, which is targeted to sulfhydryl groups on proteins. Heterobifunctional cross-linkers

†Present Address: Regeneron Pharmaceuticals, Inc., Tarrytown, NY 10591, USA

***Corresponding author:** Xiaobin Xu, PhD, Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Rd, Tarrytown, NY 10591, USA, Tel: +1-914-847-7076; E-mail: xiaobin.xu@regeneron.com

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can contain one photo-reactive group that is stable until it is exposed to high intensity UV light. Both homobifunctional cross-linkers and heterobifunctional cross-linkers can eliminate the spacer arm and mediate cross-linking between two proteins by creating a bond without an intervening linker. This type of cross-linker is termed as zero-length cross-linkers. The most widely used zero-length cross-linkers are carbodiimides, which mediate amide bond formation between a carboxylate and an amine group.

To facilitate the mass spectrometry detection and database search of cross-linked peptides, many novel cross-linking reagents have been developed, including cleavable cross-linkers, affinity-tagged cross-linkers, and isotopically tagged cross-linkers.

Placing a cleavable bond within a cross-linker structure can simplify the identification of cross-linked peptides. After cleavage, a cross-linked peptide behaves like a linear peptide with a modification, which enables the identification of cross-linked peptides using common protein database search engines. Cleavable cross-linkers can be cleaved by chemical reagents (e.g. DTT), by UV, or by tandem MS fragmentation (e.g. CID or ETD). The cleavable cross-linkers, such as Protein Interaction Reporter (PIR), have been applied for *in vivo* identification of protein-protein interactions [11]. Incorporating an affinity group into the structure of a cross-linker reagent enables the enrichment of cross-linked products. The typical affinity-tagged cross-linkers use biotin/avidin affinity purification strategies [12]. However, affinity-tagged cross-linkers are more bulky than conventional cross-linkers, which induce steric hindrance to prevent probing certain protein interactions. Alternatively, incorporation of an azide group provides a smaller cross-linker and enables click chemistry based enrichment [11]. An isotopically tagged linker substitutes one or more atoms in the cross-linker with heavy stable isotopes, usually deuterium or ^{13}C. Peptides cross-linked with a mixture of heavy and light cross-linking reagents generate unique doublet peaks in the mass spectra, which not only facilitates MS detection of cross-linked peptides and but also enables targeted MS/MS of cross-linked peptides. Isotopically tagged cross-linkers have been used to investigate complex protein interaction networks at large scale [8,9].

Cross-linking Identification Algorithms

Cross-linked peptides pose challenges to they since conventional protein database search engines were designed for identification of linear peptides or linear peptides with modifications, but not for two peptides connected with a linker. Many MS and MS/MS search algorithms have been developed for different types of cross-linkers and their applications. xComb [13], MassMatrix [14], StarvroX [15], XLink-identifier [16], and pLink [17,18] are commonly used for non-cleavable homobifunctional or heterobifunctional cross-linkers. By importing cross-linker information and protein FASTA database, xComb creates a linearized and concatenated cross-linked peptide database that can be used as a common search engine. MassMatrix is a comprehensive database search engine for MS/MS based proteomics, and it also provides algorithms for chemical cross-links identification. StavroX provides an easy-to-use graphical interface and shortens the processing time by only processing the MS/MS spectra of the precursor ions that match to the theoretical masses of cross-linked peptide candidates. MeroX [19], is designed for CID-MS/MS cleavable cross-links with a self-explanatory graphical user interface, similar to StavroX. BLinks [20] is designed for the PIR cross-linking applications. GPMAW [21] and xQuest/xProphet [8,22] can be used for identification of isotopically tagged cross-linking. GPMAW is suitable for small-scale analysis. xQuest can be used for large proteome-wide cross-linking studies [8,9]. Recently, xProphet was integrated into the xQuest to determine FDRs of large cross-linking data sets using a target-decoy strategy, which improve the scoring function and validation. XiQ [23] is designed for quantitative cross-linking mass spectrometry using heavy and light isotopically tagged cross-linkers.

Conclusion and Perspective

The applications of chemical cross-linking mass spectrometry continue to grow. One direction for chemical cross-linking mass spectrometry is to capture real time proteome-wide protein-interaction networks *in vivo*. This can be achieved by the comparison of systematic interaction changes upon a specific stimuli or perturbation using cross-linking snapshots. Some pioneer studies have been applied in bacterial cells [8,10,24]. Another direction is quantitative cross-linking. Quantitative probing protein conformational changes or protein-interaction changes can be achieved by using heavy and light isotopically tagged cross-linkers [23,25]. The continuous advancement in MS instrumentation, cross-linking reagents, and informatics tools has great potentials to enable global scale, high-throughput identification and quantification of cross-linked products, making cross-linking mass spectrometry an important technique to profile protein structures as well as deciphering dynamic protein-interaction networks *in vivo*.

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