

Review Article

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

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Protein cross-linking strategies coupled to mass spectrometry is a powerful tool for studying protein structures, protein surface topologies and protein-protein interactions. There are more than one hundred chemical crosslinkers, and some of them are already commercially available. However, the current researches require new and more effective crosslinkers and more intelligent data analysis software tools. Here, we describe some new features of crosslinkers such as cleavable cross-linkers that facilitate detection by mass spectrometry, affinity crosslinkers using click-chemistry that facilitate separation and enrichment of cross-linked peptides, and crosslinkers with the combined features. In addition, the evolution in software remarkably improves the data analysis efficiency and makes it possible to perform large-scale identification of cross-linked peptides. The advances in protein cross-linking analysis software are also addressed.

Keywords: Mass spectrometry; Chemical crosslinker; Protein cross-linking; Proteomics**Abbreviations**

MS/MS: Tandem MS; LC: Liquid Chromatography; CID: Collision Induced Dissociation; ETD: Electron Transfer Dissociation; NHS: N-hydroxysuccinimide; DSS: Disuccinimidyl Suberate; BS3: Bis[sulfosuccinimidyl]Suberate; CLIP: Click-enabled Linker for Interacting Proteins; PIR: Protein Interaction Reporter.

Introduction

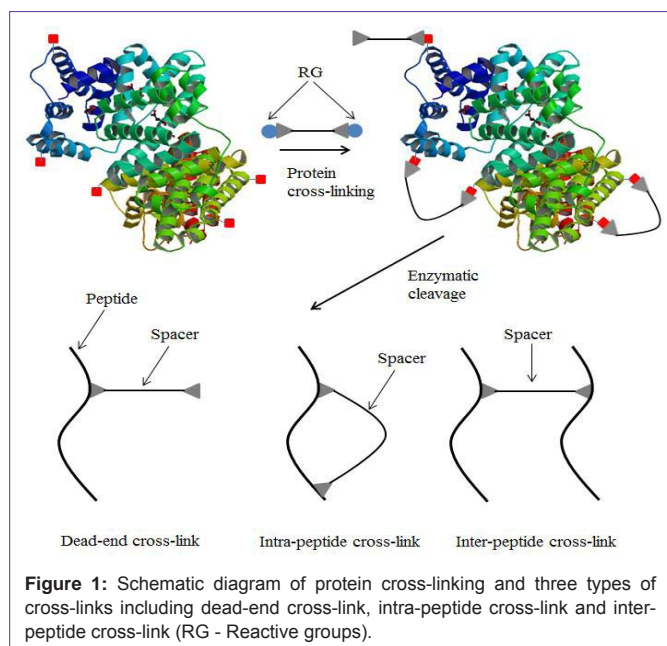
Chemical cross-linking combined with mass spectrometry can be a powerful approach for the identification of protein-protein interactions and providing information on protein structures [1-3]. This technique is emerging as an alternative to traditional structural biological methods such as time-consuming crystallography-based X-ray analyses and NMR-based approaches. The current chemical crosslinkers provide the feasibility to study proteins structures and interactions. However, the heterogeneity and low abundance of the cross-linked products continue to pose enormous challenges for large-scale biological application of cross-linking approaches [4]. Moreover, the huge amount of data collected as well as the theoretical combinatorial complexity inherent to the cross-linking process, has encouraged the scientists to develop specialized software tools to analyze mass spectra, to mine databases, and to generate pertinent structural information. In this review, we address several important issues that are related to the studies of protein structures and protein-protein interactions by cross-linking coupled to mass spectrometry analysis. Furthermore, the development of novel cross-linking reagents with some new features in recent years to optimize cross-linking strategies for mass spectrometry analysis is also described. Currently our laboratory focuses on the development of novel CID-/ETD-cleavable crosslinkers to facilitate mass spectrometry detection of cross-linked peptides as well as their application to decipher innate

immune signaling pathways of Toll-like Receptors (TLRs).

Protein cross-linking and new features of chemical crosslinkers

Cross-linking is the process of formation of covalent bonds that links one molecule to another. In biological studies, it refers to the use of a probe to link two interacting proteins in order to study protein structures and protein-protein interactions. It is used to determine the domains of proteins which are close to each other to form covalent linkage in a protein complex, and to determine the sites where the reaction occurs. In relation to the size of the crosslinkers, the data collected can be viewed as a set of distance constraints providing clues on protein structures and the topologies of multi-protein complex [5]. The major challenges in protein interaction studies with chemical cross-linking arises from the complexity of intra-, inter-, and dead-end cross-linked peptide mixtures (Figure 1)[6]. Although, there is remarkable advancement in mass spectrometry instruments in recent years but there is still a great need for effective crosslinker with innovative features as well as user-friendly data analysis software to advance this technology for large-scale applications, such as deciphering the systems-level protein signaling networks.

More than one hundred of cross-linkers have been described in the literature. Most often the targets of cross-linking functional groups are the strong nucleophiles such as the sulfhydryl and amine groups of cysteine and lysine side chains. There are varieties of crosslinkers that employ N-hydroxysuccinimide (NHS) esters for reacting with amine groups and N-maleimide for reacting with thiols. For example, disuccinimidyl suberate (DSS) and its more water-soluble sulfonate analogue bis[sulfosuccinimidyl] suberate (BS3) is a commonly used crosslinker which reacts easily with the amine groups of the proteins. In recent years, some new crosslinkers are emerging, which have



critical features, such as isotopic coding, cleavability, affinity groups, new reactive groups, and compatibility with mass spectrometry [7]. These chemical crosslinkers with new features can assist to facilitate the data acquisition and analysis by mass spectrometry.

Cleavable crosslinkers and click-based enrichment

The introduction of a cleavable bond within the crosslinker structure simplifies the identification of cross-linked peptides and data analysis after tandem mass spectrometry analysis [8]. The specific cleavable bond can be cleaved either chemically or by fragmentation with tandem mass spectrometry systems (for e.g. under CID or ETD condition), and results in unique signatures in mass spectra, which can greatly simplify data analysis. A widely used mass spectrometry-cleavable cross-linking strategy is Protein Interaction Reporter (PIR) technology, which was successfully applied for *in vivo* identification of protein-protein interactions as well as actual regions of the interacting proteins that share close proximity while present within cells [4,6]. Moreover, the purification of cross-linked peptides can be facilitated when the crosslinker contains specific affinity group. The typical affinity-based approach used in crosslinkers is the biotin-avidin affinity capture strategies. However, the drawback of affinity based crosslinkers is that they need to be custom synthesized and are generally more bulky than conventional reagents. This may affect their reactivity because of steric hindrance, and the accuracy of spatial constraints is reduced [9]. Thus, multifunctional but compact crosslinkers must be addressed.

A compact crosslinker was reported by Chowdhury et al. with two distinct features [1]. The crosslinker consists of an alkyne tag which enabled enrichment of the cross-linked peptides after proteolytic cleavage using alkyne-azido click chemistry. A small molecule detection tag (NO_2) in the crosslinker enabled the detection of neutral loss of this small NO_2 moiety as a secondary means of detecting cross-linked peptides in MS/MS analyses, which provided additional confidence in peptide identifications. The CLIP (click-enabled linker for interacting proteins) technique facilitates both

peptide enrichment and mass spectrometry data acquisition. Using CLIP, cross-linked ubiquitin peptides were successfully enriched from complex samples and the automated identification of cross-linked peptides was improved with both CID-MS/MS (collision-induced dissociation tandem mass spectrometry) and ETD-MS/MS (electron transfer dissociation) modes.

New computer software to decipher acquired MS data from cross-linked peptides

The analysis of MS data from cross-linking experiments with traditional non-cleavable crosslinkers is still a challenge because of the large number of possible combinations that have to be considered in the search algorithm. Some publicly available processing tools, such as xComb and xQuest have been developed for use with standard proteome search engine to simplify the identification of cross-linked peptides [10,11]. Other individualized bioinformatics tools were also developed to do more specialized analysis of cross-linked data [12-14]. Nevertheless, the identification of cross-links from biological protein complex is still challenging and more powerful and individualized bioinformatics tools need to be developed for the complicated calculation and assignment of the big data set from the cross-linked mass spectrometry data. Currently, there is no effective software available to study CID-cleavable cross-linking data from large-scale application.

Du et al. [15] established a comprehensive data analysis platform, Xlink-Identifier, which has been developed to support label-free analyses of data from traditional non-cleavable crosslinkers. It can identify inter-peptide, intra-peptide, and dead-end cross-links as well as underivatized peptides. The software streamlines data preprocessing, peptide scoring, and visualization and provides an overall data analysis strategy for studying protein-protein interactions and protein structures using mass spectrometry. Xlink-Identifier offers the potential to perform large-scale identifications of protein-protein interactions using tandem mass spectrometry.

Conclusions and Perspectives

Over the past two decades, mass spectrometry (MS) analysis of peptides and proteins has evolved dramatically. With the aid of chemical crosslinkers coupled to mass spectrometry, the structure information of proteins and protein-protein interactions can be studied in an accurate and efficient way. However, some technical limits still exist, for e.g. the current database is not integrated with cross-link information and special software has to be used for cross-linking analysis. The future demands to advance this research field require more intelligent analytical software tools and more versatile chemical crosslinkers with innovative features, which can identify protein signaling networks with high confidence.

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