

# Chapter 8

## Structural Investigation of Proteins and Protein Complexes by Chemical Cross-Linking/Mass Spectrometry



Christine Piotrowski and Andrea Sinz

**Abstract** During the last two decades, cross-linking combined with mass spectrometry (MS) has evolved as a valuable tool to gain structural insights into proteins and protein assemblies. Structural information is obtained by introducing covalent connections between amino acids that are in spatial proximity in proteins and protein complexes. The distance constraints imposed by the cross-linking reagent provide information on the three-dimensional arrangement of the covalently connected amino acid residues and serve as basis for *de-novo* or homology modeling approaches. As cross-linking/MS allows investigating protein 3D-structures and protein-protein interactions not only *in-vitro*, but also *in-vivo*, it is especially appealing for studying protein systems in their native environment. In this chapter, we describe the principles of cross-linking/MS and illustrate its value for investigating protein 3D-structures and for unraveling protein interaction networks.

**Keywords** Cross-linking · Mass spectrometry · Protein 3D-structure · Protein-protein interactions

### 8.1 Introduction

Proteins play pivotal roles in all biological processes. As the structure of a protein dictates its function, investigating the 3D-structure of a protein and clarifying its interactions with other proteins is one of the most important tasks to elucidate biological processes. While the 3D-structural analysis of proteins is commonly achieved by nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, and cryo-electron microscopy (cryo-EM), protein-protein interactions might be

---

C. Piotrowski · A. Sinz (✉)

Department of Pharmaceutical Chemistry & Bioanalytics, Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany  
e-mail: [andrea.sinz@pharmazie.uni-halle.de](mailto:andrea.sinz@pharmazie.uni-halle.de)

identified by co-immunoprecipitation or Förster resonance energy transfer (FRET) (Operana and Tukey 2007).

To date, NMR and X-ray crystallography are still the dominating techniques to determine high-resolution protein structures as is indicated by the large number of structures available in the PDB (~120,000 structures obtained by X-ray crystallography versus ~12,000 structures obtained by NMR spectroscopy). Limitations of both high-resolution techniques, however, persist in the investigation of very large and transient protein complexes as well as membrane proteins. Cryo-EM overcomes some of these limitations as structural analysis can be performed at rather low protein concentrations (less than 1  $\mu$ M) and highly complex protein assemblies can be targeted (Li et al. 2013).

Cross-linking/MS is an approach that complements the high-resolution 3D-structural techniques and has emerged as promising tool for the structural investigation of proteins and protein complexes in the last years (Young et al. 2000). Especially the combination of cryo-EM with cross-linking-MS has proven beneficial to provide insights into large protein assemblies (Greber et al. 2014; Weisz et al. 2017; Benda et al. 2014) that cannot be obtained by X-ray crystallography or NMR spectroscopy.

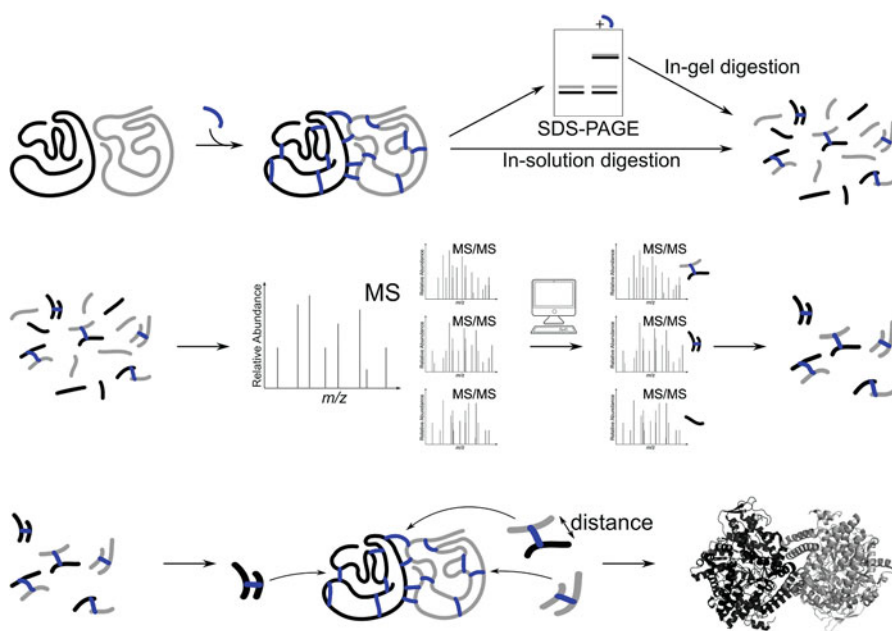
Cross-linking/MS relies on introducing covalent connections between functional groups of amino acid side chains by a chemical reagent. This cross-linker possesses a defined length and connects only these amino acids that are in the appropriate distance to be cross-linked. Usually, the analysis of the cross-linked amino acids is performed in a classical proteomics “*bottom-up*” approach where the cross-linked protein(s) are enzymatically digested and the peptide mixtures are analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS). This highly sensitive method allows examining as low as femto- to attomole amounts of proteins. The distance constraints that are derived from the cross-linked amino acids are subsequently employed for *de-novo* or homology modeling approaches (Leitner et al. 2016; Rappsilber 2011; Sinz 2014; Walzthoeni et al. 2013; Politis et al. 2014).

Importantly, the cross-linking/MS approach is not only applicable to the 3D-structural analysis of purified proteins, but it also allows elucidating protein-protein interaction networks (Häupl et al. 2016; Schweppe et al. 2017). Protein-protein interaction studies are often based on an affinity enrichment of a tagged bait protein to a specific matrix (Puig et al. 2001; Gavin et al. 2002), together with its interaction partners. Here, the washing procedure applied to remove non-interacting proteins is a crucial step, which however harbors the risk of losing transiently or weakly bound protein interaction partners. Due to the covalent fixation of proteins in the cross-linking/MS approach, the loss of weakly bound proteins during the washing procedure is circumvented.

In this chapter, we give an introduction into the principles of cross-linking/MS and present examples for successful applications of this approach to derive 3D-structural information of proteins and to identify protein interaction networks.

## 8.2 The Cross-Linking/MS Strategy

Once the 3D-structure of a protein or protein complex is covalently fixed by a cross-linking reagent *in-vitro* or *in-vivo*, the identification of the cross-linked amino acids will ultimately give insights into the spatial organization of the protein system under investigation. After the cross-linking reaction, the reaction mixture is analyzed by one-dimensional gel electrophoresis (SDS-PAGE) to visualize the result of the cross-linking reaction and to eventually optimize the reaction conditions (Sinz 2006; Rappsilber 2011) (Fig. 8.1). As mentioned above, the analysis of the cross-linked amino acids is commonly achieved by a “bottom-up” approach, including enzymatic digestion and LC/electrospray ionization (ESI)-MS/MS analysis of the resulting peptide mixture. Proteolysis is realized either by *in-gel* or *in-solution* digestion. Applying the *in-gel* approach, the band containing the protein or protein complex of interest is excised and digested within the gel. Alternatively, proteolytic cleavage can be carried out directly *in-solution* without previous separation of the proteins. The resulting peptide mixture is highly complex as it not only contains



**Fig. 8.1** Cross-linking/MS workflow. A protein or protein complex is stabilized by introducing a covalent bond with a cross-linking reagent. Separation of the cross-linked protein(s) by SDS-PAGE is followed by enzymatic *in-gel* or *in-solution* digestion, resulting in a peptide mixture containing cross-linked and non-cross-linked (linear) peptides. Applying the peptide mixture to MS analysis enables the identification of cross-linked peptides by customized software tools that match MS/MS spectra to potential cross-linking candidates. The cross-links identified provide distance information for modeling protein 3D-structures or for identifying protein interaction partners

non-cross-linked, i.e., linear, and cross-linked peptides of the target proteins, but also peptides of possible contaminants or the protease used for digestion. In subsequent LC/MS/MS analysis, mass spectra and fragment ion mass spectra are recorded, followed by the identification of cross-linked peptides by specific software tools, such as xQuest (Rinner et al. 2008), pLink (Yang et al. 2012), StavroX (Götze et al. 2012a) or Kojak (Hoopmann et al. 2015), that automatically match MS/MS spectra of cross-linked peptides to cross-linking candidates. The cross-links deliver (i) distance information for 3D-structural computational modeling and (ii) insights into the identity of protein interaction partners and protein-protein interaction sites.

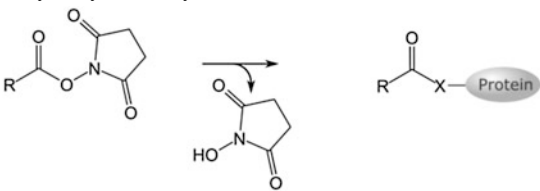
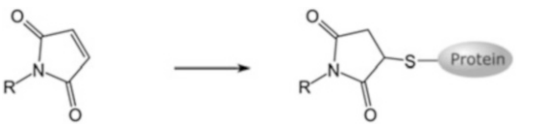
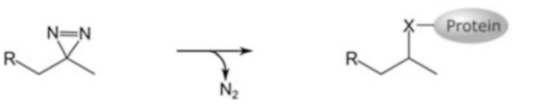
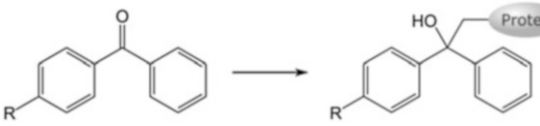
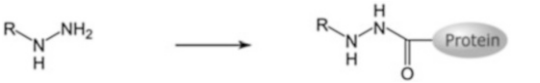
## 8.3 Experimental Design of the Cross-Linking/MS Workflow

### 8.3.1 Cross-Linker Design and Reactivity

The commonly used cross-linkers comprise two reactive head groups that are separated by a spacer with a defined length (Sinz 2006). The spacer determines the distance between the amino acids to be covalently connected and as such serves as “molecular ruler” within a protein or protein complex. Cross-linking reagents are categorized into homobifunctional cross-linkers, comprising identical head groups, or heterobifunctional cross-linkers with non-identical reactive sites (Table 8.1). The most frequently employed reactive groups are *N*-hydroxysuccinimidyl (NHS) esters targeting primary amines in lysines and protein *N*-termini. For NHS esters, an additional reactivity for hydroxy groups in serines, threonines, and tyrosines has been observed (Mädler et al. 2009; Kalkhof and Sinz 2008). Heterobifunctional linkers often contain NHS esters as one of the reactive groups (Hermanson 1996). The second reactive site can be a maleimide, targeting cysteine residues, or a photo-reactive group, such as diazirines or benzophenones. Photo-reactive moieties react in a non-specific manner, potentially connecting all 20 amino acids that are in spatial proximity. For diazirines, a preference for acidic amino acids was observed (Ziemianowicz et al. 2017; Jumper et al. 2012; Iacobucci et al. 2018), while benzophenones target mostly methionines (Wittelsberger et al. 2006). Cross-linking of acidic amino acids is still a challenging task at experimental conditions that do not interfere with the native protein structure. Hydrazines react with aspartic and glutamic acid residues as well as with the *C*-terminus of proteins upon activation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Novak and Kruppa 2008) or 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (Leitner et al. 2014a). The activated acidic amino acid might then also react with primary amines of lysines and the *N*-terminus, forming a direct connection between a carboxylic acid and an amine group (Schwarz et al. 2016).

In addition to the cross-linking reagents that are externally introduced (Table 8.1) photo-reactive, unnatural amino acids are available that are directly incorporated into proteins (Suchanek et al. 2005; Piotrowski et al. 2015). These photo-reactive

**Table 8.1** Functional groups in cross-linking reagents

Reactive group	Targeted amino acid(s)
<p><i>N</i>-hydroxysuccinimidyl (NHS) ester</p> 	Lysine, <i>N</i> -terminus, serine, threonine, tyrosine X = NH, O
<p>Maleimide</p> 	Cysteine
<p>Diazirine</p> 	All amino acids, <i>N</i> - and <i>C</i> -terminus, acidic residues (aspartate, glutamate) preferred X = CH <sub>2</sub> , NH, O or S
<p>Benzophenone</p> 	All amino acids, <i>N</i> - and <i>C</i> -terminus, methionine preferred
<p>Hydrazine</p> 	Acidic amino acids (aspartate, glutamate), <i>C</i> -terminus

An overview of commercially available cross-linking reagents is provided at <https://www.thermofisher.com/de/en/home/life-science/protein-biology/protein-labeling-crosslinking/protein-crosslinking.html>

amino acid analogues contain photo-reactive groups, such as benzophenones or diazirines, and they are incorporated into proteins during the translation process in living cells. In general, two strategies are applied, where the photo-reactive amino acids are incorporated into the protein(s) either in a site-specific or a non-directed fashion. The site-specific incorporation of the photo-reactive amino acid makes use of the amber stop codon that is placed at the desired position in the DNA. The amber stop codon encodes for the photo-reactive amino acid, e.g. *para*-benzoylphenylalanin (Bpa) (Ryu and Schultz 2006; Schwarz et al. 2016). To incorporate the photo-reactive amino acid, a specific transfer RNA (tRNA) is needed, which is encoded by an additional plasmid to be transformed or transfected into the cell. The tRNA binds the photo-reactive amino acid and incorporates it into the protein at the amber stop codon position. The major advantage of this approach is that the cross-linking reaction will specifically take place at the desired position



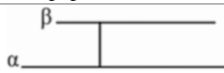
within the Bpa-labeled protein. On the other hand, the non-directed incorporation of the photo-reactive amino acid exploits the translation machinery of the cell to incorporate photo-reactive amino acids. As such, photo-methionine (photo-Met) or photo-leucine (photo-Leu) can be incorporated into proteins by the respective tRNAs for methionine and leucine (Suchanek et al. 2005; Piotrowski et al. 2015; Lössl et al. 2014; Häupl et al. 2017; Iacobucci et al. 2013). Efficient incorporation of photo-Met into proteins has been shown for different cell types (*E. coli*, HEK 293 and HeLa cells) with incorporation rates of 30–35% (Piotrowski et al. 2015). A detailed protocol of a cross-linking approach using the complementary cross-linking principles of BS<sup>2</sup>G and photo-reactive amino acids is provided in (Lössl and Sinz 2016).

### 8.3.2 Identification of Cross-Linked Peptides

As shown in the cross-linking/MS workflow, cross-linked peptides are generated by enzymatic cleavage of the cross-linked proteins by a specific protease (Fig. 8.1). The most prominent protease is trypsin that cleaves proteins C-terminally to basic amino acids (lysine and arginine residues). Cross-linking/MS however differs from the usual proteomics workflow as the use of one single protease is in some cases not sufficient. As two peptides are covalently connected, high molecular weight products are generated exceeding the optimal range of peptide MS detection. Applying a protease additionally to trypsin, such as GluC (cleaving C-terminally to glutamate and aspartate residues), will decrease the molecular weight of cross-linked peptides (Piotrowski et al. 2015). Another approach to generate peptides with lower molecular weight is to conduct proteolysis by an unspecific protease, such as proteinase K (Petrotchenko et al. 2012).

In general, cross-linked peptides are categorized into three different classes as type 0, type 1, and type 2 cross-links (Table 8.2) (Schilling et al. 2003). Type 0 (“dead-end” or “mono-link”) describes a peptide, in which one amino acid is modified by a cross-linker reagent. Here, only one reactive group of the cross-linker has reacted with an amino acid, while the other one has been hydrolyzed or has reacted with the reagent that was used for quenching the cross-linking reaction. “Dead-end” cross-links can deliver insights into the solvent-accessible surface of specific amino acids and as such, give information on the overall topology of the protein under investigation. Type 1 (intrapeptide or “loop-link”) describes the connection of two neighboring amino acids within one peptide. Only

**Table 8.2** Nomenclature of cross-linked peptides

Type 0	Type 1	Type 2
“Dead-end”, “mono-link”	Intrapeptide (“loop”-link)	Interpeptide
		

limited information on the protein's tertiary structure is provided by these cross-links. Type 2 (interpeptide) cross-links connect two peptides originating from one protein or interacting proteins. This class represents the most valuable cross-linked products that yield information on the structural proximity of specific amino acid residues and allow deducing 3D-structural information. According to the systematic nomenclature provided by Schilling et al., the higher molecular weight peptide is termed “ $\alpha$ -peptide” whereas the peptide with the lower molecular weight is referred to as “ $\beta$ -peptide” (Schilling et al. 2003).

The number of software tools for identifying cross-linked peptides from MS data is steadily increasing and not easy to review. Table 8.3 provides an overview comprising several of the so far developed software applications. The general workflow of these software tools includes an *in-silico* digestion of proteins. Subsequently, potential cross-link candidates are automatically compared to the recorded MS/MS spectra and matching cross-link candidates are reported. In addition to the software tools available for cross-link identification, an increasing number of software applications is available to further examine the cross-links, such as xVis (Grimm et al. 2015) or Xlink-DB (Zheng et al. 2013). These applications provide a visualization of the identified cross-links either in a schematic fashion

**Table 8.3** Selected software tools for identifying cross-linked peptides

Software	References
CLPM	Tang et al. (2005)
Crux	McIlwain et al. (2014)
DXMSMS	Petrotchenko et al. (2014)
ECL/ECL2	Yu et al. (2016, 2017)
FINDX	Soderberg et al. (2012)
Kojak	Hoopmann et al. (2015)
MassAI (CrossWork)	Rasmussen et al. (2011)
MassMatrix	Xu et al. (2008)
MassSpecStudio	Sarpe et al. (2016)
MS-bridge (included in USCF prospector)	<a href="http://prospector.ucsf.edu">http://prospector.ucsf.edu</a>
PeptideMap	Fenyo (1997)
pLink	Yang et al. (2012)
Pro-cross-link	Gao et al. (2006)
ProteinXXX (included in GPMAW)	Nielsen et al. (2007)
SIM-XL	Lima et al. (2015)
StavroX/MeroX	Götze et al. (2012b, 2015)
Xi	Fischer et al. (2013) and Giese et al. (2016b)
Xilmass	Yilmaz et al. (2016)
Xlink analyzer	Kosinski et al. (2015)
Xlink-identifier	Du et al. (2011)
XlinkX/XlinkX 2.0	Liu et al. (2015) and Liu et al. (2017)
XLPM	Jaiswal et al. (2014)
xComb	Panchaud et al. (2010)
xQuest	Rinner et al. (2008)

or by mapping them into published PDB structures. Also, there are software tools available for quantifying cross-linked peptides, e.g. xTract (Walzthoeni et al. 2015) or XiQ (Fischer et al. 2013).

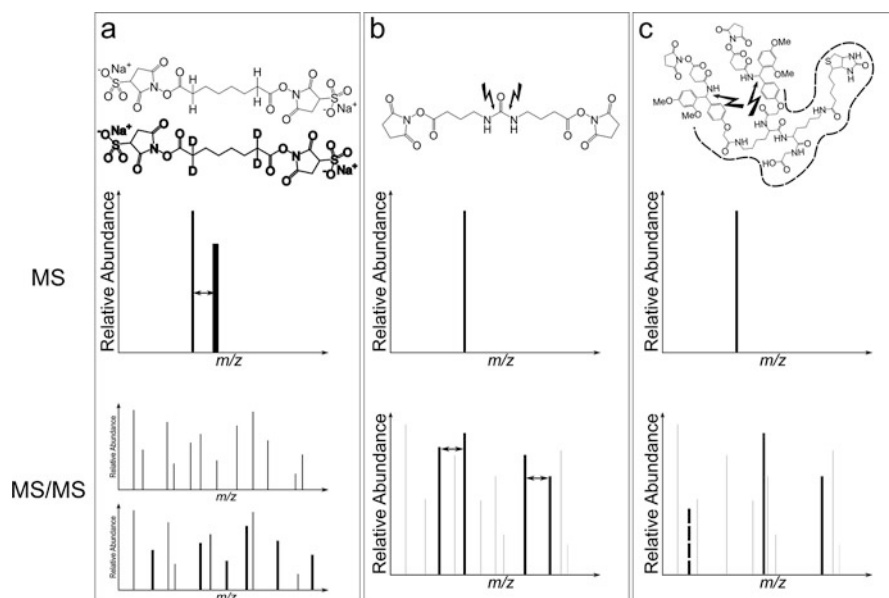
### 8.3.3 *Facilitated Analysis of Cross-Linked Products*

The identification of cross-linked products is still challenging due to their low abundance in the peptide mixtures generated after enzymatic digestion (Fig. 8.1). Specifically, we see two main difficulties: (i) Cross-linked peptides might be missed during MS analysis, (ii) false-positive identifications of cross-links might occur due to their great variability. To overcome these problems, sample complexity can be reduced or cross-linked products can be enriched. Alternatively, cross-linkers carry an isotope signature (usually by introducing deuterium atoms) or create specific fragment ion patterns during MS/MS experiments for automated data analysis.

Strong cation exchange (SCX) and size-exclusion chromatography (SEC) are the methods of choice to enrich cross-linked peptides (Leitner et al. 2010, 2014b; Schmidt and Sinz 2017). Also, an affinity enrichment of cross-linked products is based on a biotin tag that specifically binds to avidin. Biotin is either incorporated in the cross-linking reagent (Tang and Bruce 2010) or is introduced after the cross-linking reaction by click chemistry (Nury et al. 2015). In order to utilize SCX enrichment of cross-linked peptides, enzymatic cleavage has to be performed with a protease cleaving C-terminally to basic amino acids, such as trypsin. Consequently, every peptide carries a positive charge at the C-terminus that can be utilized for SCX enrichment. As cross-linked products are composed of two peptides, they accommodate a higher number of positive charges than linear peptides. Thus, the cross-linked peptides can be enriched by SCX (Leitner et al. 2010; Fritzsche et al. 2012; Schmidt and Sinz 2017; Tinnefeld et al. 2017). As cross-linked peptides usually possess higher molecular weights than non-cross-linked peptides, they can also be enrichment by SEC (Herzog et al. 2012; Rampler et al. 2015). A major advantage of SEC is its applicability for all peptide mixtures, independent of the protease used for digestion, but on the other hand, low-molecular weight cross-linked peptides might get lost during SEC enrichment.

For an unambiguous identification of cross-linked products, cross-linking reagents with unique characteristics have been designed. The first class of cross-linkers contains isotope labels, in most cases deuterium atoms, such as bis(sulfosuccinimidyl)suberate (BS<sup>3</sup>) D<sub>0</sub>/D<sub>4</sub> (Fig. 8.2a) (Müller et al. 2001; Schmidt et al. 2005). The deuterated and non-deuterated version of the cross-linker are mixed in a 1:1 ratio and added to the protein solution. Hence, every cross-linked product is visible in mass spectra as a specific doublet of signals. Both species generate nearly identical MS/MS spectra, but differ in the fragment ions containing the deuterated or non-deuterated cross-linker. Acquiring MS/MS spectra from both isotope species helps in unambiguously identifying cross-linked peptides as two spectra with the specific mass shift are only obtained for cross-linked products, but not for linear peptides.





**Fig. 8.2** Strategies to facilitate the analysis of cross-linked peptides. Structures of respective cross-linkers are presented in the upper panel. Specific cleavage sites of the cross-linkers upon collisional activation inside the mass spectrometer are indicated. The middle panel (MS) displays how the cross-linked peptides appear in the mass spectrum, while the lower panel (MS/MS) shows characteristics of the cross-linked products in fragment ion mass spectra. **(a)** For the isotope-labeled cross-linker  $BS^3(D_0/D_4)$ , two signals are detected in the mass spectrum for one cross-linked peptide pair differing by the isotope label (4 amu). Subsequently, two MS/MS spectra for one cross-linked peptide pair are recorded, black –  $BS^3D_0$ ; bold –  $BS^3D_4$ . **(b)** For the MS-cleavable cross-linker DSBU, characteristic fragment ion signatures of the linker (two doublets) are visible in MS/MS spectra. **(c)** PIR cross-linkers exhibit characteristic fragment ions (dashed line) and peptides modified with cross-linker fragments (bold lines) in MS/MS spectra

A highly attractive approach that is currently gaining more and more importance is employing cross-linkers with an MS-cleavable moiety (Sinz 2017). The cross-linker is cleaved during collisional activation in the gas phase inside the mass spectrometer resulting in specific fragment ions that contain parts of the cross-linker. In Fig. 8.2b, the MS-cleavable cross-linker disuccinimidyl dibutyric urea (DSBU, formerly BuUrBu) is presented. DSBU comprises two NHS esters as reactive sites and a cleavable urea moiety (Müller et al. 2010). After fragmentation of the linker, two doublet signals are visible for each interpeptide (type 2) cross-linked product in the MS/MS spectrum. These doublets result from cleavage of one of the two NH–CO bonds of the urea moiety. Thus, two pairs of asymmetric fragments are generated, exhibiting a specific mass difference of 25.979 amu that allows an unambiguous identification of a cross-linked product.

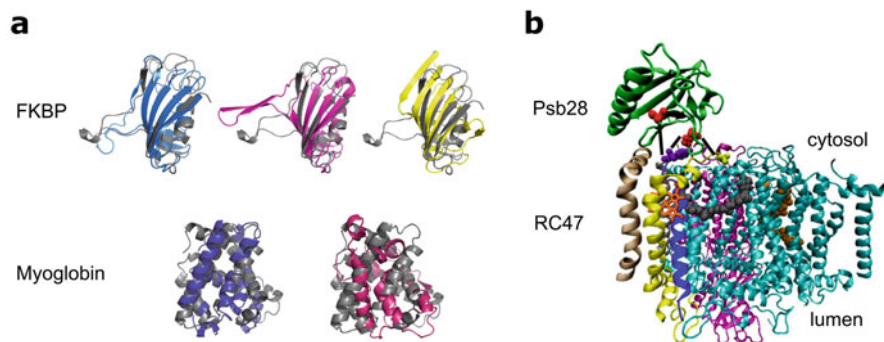
There are other MS-cleavable cross-linkers available that generate characteristic fragment ions, such as a class of reagents termed “protein interaction reporters

(PIR)” (Fig. 8.2c) (Tang and Bruce 2010). PIR cross-linkers comprise two cleavage sites releasing a specific part of the cross-linker after collisional activation inside the mass spectrometer. This specific fragment ion as well as peptides containing the remaining fragment of the cross-linker are present in every MS/MS spectrum of a cross-linked product and allow its unambiguous assignment.

## 8.4 Structural Investigation of Purified Proteins and Large Protein Assemblies

As outlined above, the distance constraints derived from the cross-linked amino acids serve as basis for a computational modeling of purified proteins or protein complexes. The distance constraints are provided as  $C_{\alpha}$ - $C_{\alpha}$  or  $C_{\beta}$ - $C_{\beta}$  distances and defined by the length of the side chains of the cross-linked residues plus the cross-linker spacer length (Merkley et al. 2014; Hofmann et al. 2015). In principle, two strategies are employed to implement these constraints into the modeling process: (i) modeling of protein structures using the cross-linking distances as input, (ii) filtering the theoretical models by the experimentally determined cross-linking distances. A number of different software tools are available for modeling protein structures, such as ROSETTA (Kaufmann et al. 2010), I-TASSER (Zhang 2009), PEP-FOLD (Maupetit et al. 2009) or Abalone (<http://www.biomolecular-modeling.com/Abalone/index.html>). The optimal cross-linker spacer lengths for protein modeling have been evaluated by combining and analyzing simulated and experimentally observed cross-linking constraints for various proteins (Hofmann et al. 2015). A specific equation was developed to predict the ideal spacer length in correlation to the size of the targeted protein. As a result, the optimal spacer length of a cross-linker to study the 35-kDa human phosphatase activator protein (PDB-ID: 2IXM) was determined to be 12.5 Å (Hofmann et al. 2015).

The investigation of a purified protein aims at elucidating its tertiary structure and the assembly state of eventually present oligomeric forms. Furthermore, newly developed cross-linking reagents are usually evaluated using small proteins, such as myoglobin, human serum albumin or bovine serum albumin (Brodie et al. 2017; Belsom et al. 2016, 2017; Giese et al. 2016a; Iacobucci et al. 2017). Small proteins, such as myoglobin and the FK506 binding protein (FKBP), have also been used for studying the impact of cross-linking applied to structural modeling with a discrete molecular dynamics (DMD) simulation based on cross-linking constraints (Brodie et al. 2017). Five short-range cross-linkers with various functional groups targeting different amino acids were employed to derive distance constraints of both proteins. The cross-linking data obtained were subsequently used as input for the DMD. Clustering of the generated models identified three clusters for FKBP and two for myoglobin. Representative model structures of each cluster were similar to the known PDB structures (Fig. 8.3a), underlining the strength of the cross-linking/MS approach to obtain native-like conformations.



**Fig. 8.3** Structural investigation of single proteins and a large protein complex. **(a)** Comparison of cross-link-based modeled structures to available PDB structures of FK506 binding protein (FKBP) and myoglobin. The best scored structures of the largest clusters are superimposed with the PDB structures (dark grey). Figure is adapted with permission from (Brodie et al. 2017). **(b)** Structure of Psb28 docked to the RC47 subcomplex of the photosystem II of the cyanobacterium *Synechocystis* sp. (Figure is adapted with permission from Weisz et al. 2017)

Applying cross-linking to large protein complexes illustrates that the size of the complexes of interest is unrestricted for cross-linking/MS approaches, while NMR or X-ray crystallography are limited in protein size by sample preparation and data acquisition. Cross-linking/MS is able to deliver structural information on small protein assemblies, such as nidogen-1/laminin  $\gamma$ 1 (Lössl et al. 2014) or chaperone Hsc70/ $\alpha$ -synuclein complexes (Nury et al. 2015), but also on large protein systems, such as the mammalian mitochondrial ribosome (Greber et al. 2014) or the ribosome post-recycling complex (Kiosze-Becker et al. 2016).

To simplify the study of protein-protein interactions, representative peptides can be employed that harbor known interaction sites, as predicted by preceding biochemical studies or computational approaches. As an example, Munc13 peptides containing the respective calmodulin (CaM) binding site were synthesized to investigate presynaptic CaM/Munc13 complexes (Dimova et al. 2009; Lipstein et al. 2012). For this, the unnatural photo-reactive amino acid Bpa was incorporated during peptide synthesis into Munc13 peptides and the peptides were applied for photo-cross-linking experiments. Additionally, the heterobifunctional cross-linker *N*-succinimidyl-*p*-benzoyldihydrocinamate (SBC), containing a NHS ester and a benzophenone group, as well as BS<sup>3</sup> and bis(sulfosuccinimidyl)-2,2,4,4-glutarate (BS<sup>2</sup>G) were applied to obtain complementary cross-linking data on the interaction between CaM and Munc13 peptides. The cross-linking constraints were then subjected to computational modeling of the CaM/Munc13 peptide complexes using the PatchDock and ROSETTADock software applications. The resulting structures revealed all Munc13 isoforms to bind similarly to CaM, indicating a common CaM-binding motif of all four Munc13 isoforms (Dimova et al. 2009; Lipstein et al. 2012).

Photosystem II intermediate complexes from cyanobacterium *Synechocystis* sp present an impressive example for applying cross-linking/MS to large protein

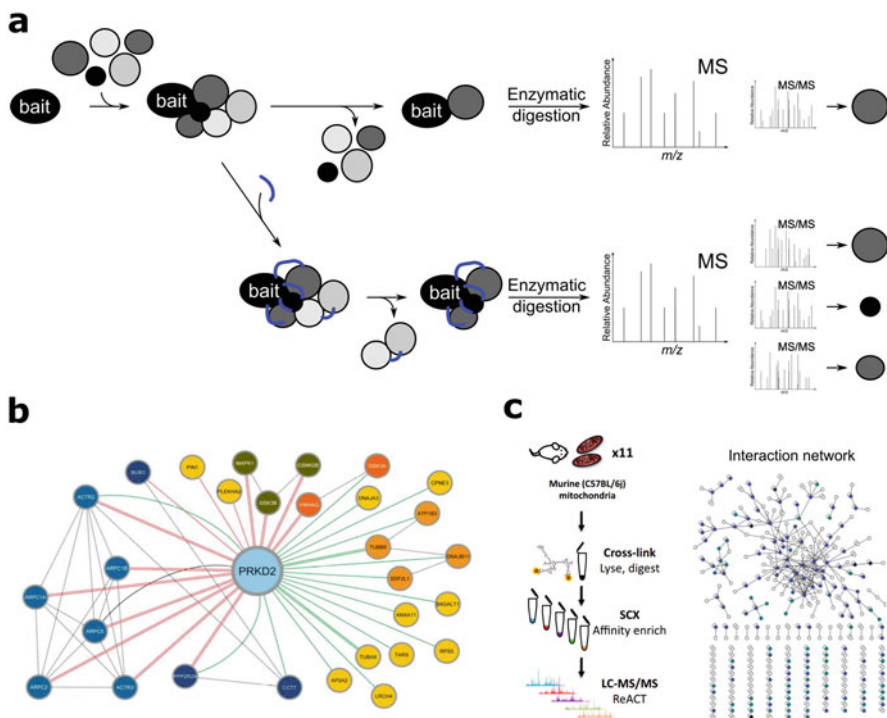
assemblies (Weisz et al. 2017). To study the interaction of the cytoplasmic photosystem II protein Psb28 with the membrane bound photosystem II component, complete photosystem II was purified from *Synechocystis sp.* Structural analysis was carried out with the homobifunctional, amine-reactive cross-linking reagent BS<sup>3</sup> ( $D_0/D_{12}$ ). The cross-linking data revealed an interaction of Psb28 with cytochrome b559, PsbE, and PsbF subunits of the photosystem II included in the RC47 subcomplex. Subsequent docking of Psb28 to the RC47 subcomplex of the photosystem II, which is known to interact with Psb28, was performed with the DOT 2.0 docking software. The resulting structures were validated by comparing the cross-linking data with the generated models. The final model of the protein complex is displayed in Fig. 8.3b, showing that Psb28 is interacting with PsbE and PsbF of the RC47 subcomplex as confirmed by the cross-linking data.

Currently, the majority of cross-linking studies is performed *in-vitro* as these studies allow a specific targeting of the proteins of interest. Deriving structural information on protein and protein complexes *in-vivo* is still a daunting task due to the enormous complexity of cellular samples. In many studies, cross-linking experiments are combined with immunoblotting. Examples include studies of the COX-2/mPGES complex and TS3-regulating proteins LcrG and LcrV (Henderson and Nilles 2017) as well as investigating the assembly state of  $\alpha$ -synuclein (Corbille et al. 2016). The analysis of  $\alpha$ -synuclein assemblies in living cells was performed by disuccinimidylglutarate (DSG) and dithiobis(succinimidyl)propionate (DSP). Both cross-linkers are NHS esters targeting amine groups in proteins, but they differ in spacer length. Additionally, the cross-linker DSP contains a disulfide bond that can be cleaved under reducing conditions (Corbille et al. 2016). Cross-linking was induced by adding the cross-linkers directly to the cell suspension, followed by disruption of the cells. Analysis of the complexes was then performed by immunoblotting using an anti- $\alpha$ -synuclein antibody revealing the presence of  $\alpha$ -synuclein dimers and pentamers in the cells. This result was verified by the cleavable DSP cross-linker as the pentamer disappeared after reduction of the disulfide bond in DSP.

## 8.5 Identification of Protein-Protein Interaction Networks

The identification of protein-protein interaction networks is the key to understanding biological processes and cross-linking/MS can make here major contributions by identifying interacting proteins as well as defining their interaction sites. Often, the result of the cross-linking reaction is monitored via SDS-PAGE or immunoblotting, giving insights only into these interaction partners for which antibodies are available (Hetu et al. 2008; Maadi et al. 2017; Henderson and Nilles 2017). The combination of cross-linking with MS will give more detailed insights, giving a more comprehensive picture on protein interaction networks.

*In-vitro* MS based procedures usually include an affinity-based identification of protein interaction partners. The starting point for these studies is the immobilization



**Fig. 8.4** Elucidation of protein-protein interaction networks. **(a)** The immobilized bait protein is incubated with a cell lysate, followed by a washing procedure to remove the non-binding proteins. LC/MS/MS analysis identifies the interacting proteins. Upper panel: common affinity-based strategy, lower panel: cross-linking-based strategy. **(b)** Identified interaction partners of protein kinase D2 applying BS<sup>2</sup>G to capture interacting proteins by the strategy presented in a) lower panel. Figure is adapted with permission from (Häupl et al. 2016). **(c)** Workflow for the identification of protein-protein interaction network from murine mitochondria. Circles display proteins, lines indicate cross-links. The color depth of each dot is proportional to the frequency of the respective protein within the eleven samples. (Figure is adapted with permission from Schweppe et al. 2017)

of a bait protein on a matrix, followed by incubation with cell lysates or cellular fractions. Several washing steps are performed to remove non-interacting proteins, followed by enzymatic digestion to identify protein binding partners (Fig. 8.4a, upper panel). Unfortunately, the washing procedure can prevent the detection of weakly or transiently bound protein-protein interaction. Applying a cross-linking reagent for a covalent fixation of interacting proteins prior to the washing procedure prevents losing potential protein binding partners (Fig. 8.4a, lower panel). Afterwards, the enriched interacting proteins are enzymatically digested and analyzed by LC/MS/MS. Cross-linked peptides as well as non-cross-linked peptides are used to identify the interaction partners. Non-cross-linked peptides identify the binding proteins, while cross-linked peptides additionally yield information on the protein interfaces.

A combined affinity purification cross-linking/MS strategy was applied to identify partners of protein kinase D2 (PKD2) from Golgi preparations and whole cell lysates. For these studies, the external cross-linker BS<sup>2</sup>G ( $D_0/D_4$ ) as well as the unnatural amino acids, photo-Leu and photo-Met, incorporated into proteins during translation in HeLa cells, were applied (Häupl et al. 2016, 2017). To investigate PKD2 interaction partners, glutathione-S-transferase (GST)-tagged PKD2 was immobilized on GSH sepharose beads and incubated with cell lysate or a Golgi preparation. PKD2-interacting proteins were covalently bound by adding the amine-reactive cross-linker BS<sup>2</sup>G ( $D_0/D_4$ ) to the reaction mixture or by inducing photo-cross-linking of the unnatural amino acids by UV-A irradiation. LC/MS/MS analysis allowed identifying the covalently fixed PKD2 interaction partners. The results obtained by the BS<sup>2</sup>G cross-linking are illustrated in Fig. 8.4b (Häupl et al. 2016). With the photo-reactive amino acids, similar PKD2 interaction partners were identified, but the complementary reactivity and shorter spacer length of the photo-reactive amino revealed additional interacting proteins (Häupl et al. 2017). A similar approach, exclusively based on BS<sup>2</sup>G ( $D_0/D_4$ ), was employed for investigating protein interaction partners of tissue-type plasminogen activator (t-PA), an established tumor marker in various cancers (Bosse et al. 2016). Proteins secreted by erlotinib-sensitive (PC9) and erlotinib-resistant (PC9ER) non-small cell lung cancer (NSCLC) cells were investigated, indicating differences of t-PA interacting proteins between erlotinib-sensitive and -resistant cells.

To enable protein interaction partner studies *in-vivo*, cross-linking reagents are added to cell cultures or cell suspensions, which are crossing the cell membrane to react with target proteins within the cell (Weisbrod et al. 2013; de Jong et al. 2017). In case of photo-reactive amino acid incorporation, the reactive groups enabling cross-linking reactions are already incorporated during cell growth. Desired cross-linking is afterwards induced by exposure of the cells to UV-A light (Yang et al. 2016a). Subsequent proteolysis of whole cells or cell lysates results in enormously complex peptide mixtures that hamper a thorough identification of cross-linked peptides. Consequently, an enrichment of cross-linked peptides is mandatory before performing LC/MS/MS analyses. Affinity strategies can be applied if the protein of interest contains a tag for the specific isolation of the desired protein complexes (Walker-Gray et al. 2017). Alternatively, the biotin label is contained in the cross-linker. As described above, the biotin label can either be incorporated in the cross-linker itself (Tang and Bruce 2010; Tan et al. 2016; Yang et al. 2016b) or it is added after to the cross-linking reaction by a click-reaction (Nury et al. 2015). The latter approach is based on orthogonal chemistry strategies developed for proteomic analyses (Speers and Cravatt 2005; Weerapana et al. 2007).

Abovementioned PIR cross-linkers (Fig. 8.2c) have been used for *in-vivo* studies identifying protein-protein interactions in mitochondria – cell organelles that are comprised of more than 1000 proteins (Schweppe et al. 2017). Cross-linking experiments were conducted on active mitochondria isolated from mouse heart and allowed the identification of protein interaction partners as well as the 3D-structural investigation of the respective protein complexes. The PIR cross-linker used for this study was membrane-permeable, and comprised two NHS esters as reactive

head groups as well as a biotin group for an enrichment of cross-links (Fig. 8.4c). After the cross-linking reaction, mitochondria were disrupted and the proteins were enzymatically digested. Cross-linked peptides were first fractionated by SCX and further enriched by affinity chromatography. Subsequent LC/MS/MS analysis by identified 327 proteins and 2427 cross-linked peptides, which additionally allowed gaining insights into the 3D-structures of selected protein complexes.

## 8.6 Conclusion

Cross-linking/MS has matured as a valuable technique in structural biology that complements existing techniques, such as X-ray crystallography, NMR spectroscopy, and cryo-EM. The major applications of the cross-linking/MS approach are to derive 3D-structural information of purified proteins and protein complexes, providing distance information for computational modeling studies, and to elucidate protein-protein interaction networks from cell lysates or even in intact cells. Although cross-linking of proteins *in-vivo* is still a challenging task, innovative approaches have been developed and are continuously being improved. A comprehensive analysis of protein-protein interaction networks in the cellular environment has become feasible.

**Acknowledgments** AS acknowledges financial support by the DFG (project Si 867/15-2) and the region of Saxony-Anhalt.

## References

- Belsom A, Schneider M, Fischer L, Brock O, Rappsilber J (2016) Serum albumin domain structures in human blood serum by mass spectrometry and computational biology. *Mol Cell Proteomics* 15(3):1105–1116. <https://doi.org/10.1074/mcp.M115.048504>
- Belsom A, Mudd G, Giese S, Auer M, Rappsilber J (2017) Complementary benzophenone cross-linking/mass spectrometry photochemistry. *Anal Chem* 89(10):5319–5324. <https://doi.org/10.1021/acs.analchem.6b04938>
- Benda C, Ebert J, Scheltema RA, Schiller HB, Baumgartner M, Bonneau F, Mann M, Conti E (2014) Structural model of a CRISPR RNA-silencing complex reveals the RNA-target cleavage activity in Cmr4. *Mol Cell* 56(1):43–54. <https://doi.org/10.1016/j.molcel.2014.09.002>
- Bosse K, Haneder S, Arlt C, Ihling CH, Seufferlein T, Sinz A (2016) Mass spectrometry-based secretome analysis of non-small cell lung cancer cell lines. *Proteomics* 16(21):2801–2814. <https://doi.org/10.1002/pmic.201600297>
- Brodie NI, Popov KI, Petrotchenko EV, Dokholyan NV, Borchers CH (2017) Solving protein structures using short-distance cross-linking constraints as a guide for discrete molecular dynamics simulations. *Sci Adv* 3(7):e1700479. <https://doi.org/10.1126/sciadv.1700479>
- Corbille AG, Neunlist M, Derkinderen P (2016) Cross-linking for the analysis of alpha-synuclein in the enteric nervous system. *J Neurochem* 139(5):839–847. <https://doi.org/10.1111/jnc.13845>
- de Jong L, de Koning EA, Roseboom W, Buncherd H, Wanner MJ, Dapic I, Jansen PJ, van Maarseveen JH, Corthals GL, Lewis PJ, Hamoen LW, de Koster CG (2017) In-culture cross-linking of bacterial cells reveals large-scale dynamic protein-protein interactions at the peptide level. *J Proteome Res* 16(7):2457–2471. <https://doi.org/10.1021/acs.jproteome.7b00068>

- Dimova K, Kalkhof S, Pottratz I, Ihling C, Rodriguez-Castaneda F, Liepold T, Griesinger C, Brose N, Sinz A, Jahn O (2009) Structural insights into the calmodulin-Munc13 interaction obtained by cross-linking and mass spectrometry. *Biochemistry* 48(25):5908–5921. <https://doi.org/10.1021/bi900300r>
- Du X, Chowdhury SM, Manes NP, Wu S, Mayer MU, Adkins JN, Anderson GA, Smith RD (2011) Xlink-identifier: an automated data analysis platform for confident identifications of chemically cross-linked peptides using tandem mass spectrometry. *J Proteome Res* 10(3):923–931. <https://doi.org/10.1021/pr100848a>
- Fenyo D (1997) A software tool for the analysis of mass spectrometric disulfide mapping experiments. *Comp Appl Biosci* 13(6):617–618
- Fischer L, Chen ZA, Rappsilber J (2013) Quantitative cross-linking/mass spectrometry using isotope-labelled cross-linkers. *J Proteome* 88:120–128. <https://doi.org/10.1016/j.jprot.2013.03.005>
- Fritzsche R, Ihling CH, Gotze M, Sinz A (2012) Optimizing the enrichment of cross-linked products for mass spectrometric protein analysis. *Rap Commun Mass Spectrom* 26(6):653–658. <https://doi.org/10.1002/Rcm.6150>
- Gao Q, Xue S, Doneanu CE, Shaffer SA, Goodlett DR, Nelson SD (2006) Pro-CrossLink. Software tool for protein cross-linking and mass spectrometry. *Anal Chem* 78(7):2145–2149. <https://doi.org/10.1021/ac051339c>
- Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, Remor M, Hofert C, Schelder M, Brajenovic M, Ruffner H, Merino A, Klein K, Hudak M, Dickson D, Rudi T, Gnau V, Bauch A, Bastuck S, Huhse B, Leutwein C, Heurtier MA, Copley RR, Edelman A, Querfurth E, Rybin V, Drewes G, Raida M, Bouwmeester T, Bork P, Seraphin B, Kuster B, Neubauer G, Superti-Furga G (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* 415(6868):141–147. <https://doi.org/10.1038/415141a>
- Giese SH, Belsom A, Rappsilber J (2016a) Optimized fragmentation regime for Diazirine photo-cross-linked peptides. *Anal Chem* 88(16):8239–8247. <https://doi.org/10.1021/acs.analchem.6b02082>
- Giese SH, Fischer L, Rappsilber J (2016b) A study into the collision-induced dissociation (CID) behavior of cross-linked peptides. *Mol Cell Proteomics* 15(3):1094–1104. <https://doi.org/10.1074/mcp.M115.049296>
- Götze M, Pettelkau J, Schaks S, Bosse K, Ihling CH, Krauth F, Fritzsche R, Kuhn U, Sinz A (2012a) StavroX—a software for analyzing crosslinked products in protein interaction studies. *J Am Soc Mass Spectrom* 23(1):76–87. <https://doi.org/10.1007/s13361-011-0261-2>
- Götze M, Pettelkau J, Schaks S, Bosse K, Ihling CH, Krauth F, Fritzsche R, Kühn U, Sinz A (2012b) StavroX-A software for analyzing crosslinked products in protein interaction studies. *J Am Soc Mass Spectrom* 23(1):76–87. <https://doi.org/10.1007/s13361-011-0261-2>
- Götze M, Pettelkau J, Fritzsche R, Ihling CH, Schafer M, Sinz A (2015) Automated assignment of MS/MS cleavable cross-links in protein 3D-structure analysis. *J Am Soc Mass Spectrom* 26(1):83–97. <https://doi.org/10.1007/s13361-014-1001-1>
- Greber BJ, Boehringer D, Leitner A, Bieri P, Voigts-Hoffmann F, Erzberger JP, Leibundgut M, Aebersold R, Ban N (2014) Architecture of the large subunit of the mammalian mitochondrial ribosome. *Nature* 505(7484):515–519. <https://doi.org/10.1038/nature12890>
- Grimm M, Zimniak T, Kahraman A, Herzog F (2015) xVis: a web server for the schematic visualization and interpretation of crosslink-derived spatial restraints. *Nucleic Acids Res* 43(W1):W362–W369. <https://doi.org/10.1093/nar/gkv463>
- Häupl B, Ihling CH, Sinz A (2016) Protein interaction network of human protein kinase D2 revealed by chemical cross-linking/mass spectrometry. *J Proteome Res* 15:3686–3699. <https://doi.org/10.1021/acs.jproteome.6b00513>
- Häupl B, Ihling CH, Sinz A (2017) Combining affinity enrichment, cross-linking with photo-amino acids, and mass spectrometry for probing protein kinase D2 interactions. *Proteomics*. <https://doi.org/10.1002/pmic.201600459>



- Henderson TA, Nilles ML (2017) In vivo photo-cross-linking to study T3S interactions demonstrated using the *Yersinia pestis* T3S system. *Methods Mol Biol* 1531:47–60. [https://doi.org/10.1007/978-1-4939-6649-3\\_4](https://doi.org/10.1007/978-1-4939-6649-3_4)
- Hermanson GT (1996) Bioconjugate techniques. Academic, San Diego
- Herzog F, Kahraman A, Boehringer D, Mak R, Bracher A, Walzthoeni T, Leitner A, Beck M, Hartl FU, Ban N, Malmstrom L, Aebersold R (2012) Structural probing of a protein phosphatase 2A network by chemical cross-linking and mass spectrometry. *Science* 337(6100):1348–1352. <https://doi.org/10.1126/science.1221483>
- Hetu PO, Ouellet M, Falguyet JP, Ramachandran C, Robichaud J, Zamboni R, Riendeau D (2008) Photo-crosslinking of proteins in intact cells reveals a dimeric structure of cyclooxygenase-2 and an inhibitor-sensitive oligomeric structure of microsomal prostaglandin E2 synthase-1. *Arch Biochem Biophys* 477(1):155–162. <https://doi.org/10.1016/j.abb.2008.04.038>
- Hofmann T, Fischer AW, Meiler J, Kalkhof S (2015) Protein structure prediction guided by crosslinking restraints – a systematic evaluation of the impact of the crosslinking spacer length. *Methods* 89:79–90. <https://doi.org/10.1016/j.ymeth.2015.05.014>
- Hoopmann MR, Zelter A, Johnson RS, Riffle M, MacCoss MJ, Davis TN, Moritz RL (2015) Kojak: efficient analysis of chemically cross-linked protein complexes. *J Proteome Res* 14(5):2190–2198. <https://doi.org/10.1021/pr501321h>
- Iacobucci C, Reale S, De Angelis F (2013) Photoactivable amino acid bioisosteres and mass spectrometry: snapshots of in vivo 3D protein structures. *Chembiochem Eur J Chem Biol* 14(2):181–183. <https://doi.org/10.1002/cbic.201200742>
- Iacobucci C, Hage C, Schafer M, Sinz A (2017) A novel MS-cleavable azo cross-linker for peptide structure analysis by free radical initiated peptide sequencing (FRIPS). *J Am Soc Mass Spectrom* 28:2039–2053. <https://doi.org/10.1007/s13361-017-1744-6>
- Iacobucci C, Götz M, Piotrowski C, Arlt C, Rehkamp A, Ihling C, Hage C, Sinz A (2018) Carboxyl- and photo-reactive, MS-cleavable cross-linkers: unveiling the real nature of diazirine-based reagents. *Anal Chem* 90(4):2805–2809
- Jaiswal M, Crabtree N, Bauer MA, Hall R, Raney KD, Zybilov BL (2014) XLPM: efficient algorithm for the analysis of protein-protein contacts using chemical cross-linking mass spectrometry. *BMC bioinformatics* 15 Suppl 11:S16. <https://doi.org/10.1186/1471-2105-15-S11-S16>
- Jumper CC, Bomgardner R, Rogers J, Etienne C, Schriemer DC (2012) High-resolution mapping of carbene-based protein footprints. *Anal Chem* 84(10):4411–4418. <https://doi.org/10.1021/ac300120z>
- Kalkhof S, Sinz A (2008) Chances and pitfalls of chemical cross-linking with amine-reactive N-hydroxysuccinimide esters. *Anal Bioanal Chem* 392(1–2):305–312. <https://doi.org/10.1007/s00216-008-2231-5>
- Kaufmann KW, Lemmon GH, Deluca SL, Sheehan JH, Meiler J (2010) Practically useful: what the Rosetta protein modeling suite can do for you. *Biochemistry* 49(14):2987–2998. <https://doi.org/10.1021/bi902153g>
- Kiosze-Becker K, Ori A, Gerovac M, Heuer A, Nurenbeg-Goloub E, Rashid UJ, Becker T, Beckmann R, Beck M, Tampe R (2016) Structure of the ribosome post-recycling complex probed by chemical cross-linking and mass spectrometry. *Nat Commun* 7:13248. <https://doi.org/10.1038/ncomms13248>
- Kosinski J, von Appen A, Ori A, Karius K, Muller CW, Beck M (2015) Xlink analyzer: software for analysis and visualization of cross-linking data in the context of three-dimensional structures. *J Struct Biol* 189(3):177–183. <https://doi.org/10.1016/j.jsb.2015.01.014>
- Leitner A, Walzthoeni T, Kahraman A, Herzog F, Rinner O, Beck M, Aebersold R (2010) Probing native protein structures by chemical cross-linking, mass spectrometry, and bioinformatics. *Molecular & cellular proteomics : MCP* 9(8):1634–1649. <https://doi.org/10.1074/mcp.R000001-MCP201>
- Leitner A, Joachimiak LA, Unverdorben P, Walzthoeni T, Frydman J, Forster F, Aebersold R (2014a) Chemical cross-linking/mass spectrometry targeting acidic residues in proteins and protein complexes. *Proc Natl Acad Sci U S A* 111(26):9455–9460. <https://doi.org/10.1073/pnas.1320298111>

- Leitner A, Walzthoeni T, Aebersold R (2014b) Lysine-specific chemical cross-linking of protein complexes and identification of cross-linking sites using LC-MS/MS and the xQuest/xProphet software pipeline. *Nat Protoc* 9(1):120–137. <https://doi.org/10.1038/nprot.2013.168>
- Leitner A, Faini N, Stengel F, Aebersold R (2016) Crosslinking and mass spectrometry: an integrated technology to understand the structure and function of molecular machines. *Trends Biochem Sci* 41(1):20–32. <https://doi.org/10.1016/j.tibs.2015.10.008>
- Li X, Mooney P, Zheng S, Booth CR, Braunfeld MB, Gubbens S, Agard DA, Cheng Y (2013) Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat Methods* 10(6):584–590. <https://doi.org/10.1038/nmeth.2472>
- Lima DB, de Lima TB, Balbuena TS, Neves-Ferreira AGC, Barbosa VC, Gozzo FC, Carvalho PC (2015) SIM-XL: a powerful and user-friendly tool for peptide cross-linking analysis. *J Proteome* 129:51–55. <https://doi.org/10.1016/j.jprot.2015.01.013>
- Lipstein N, Schaks S, Dimova K, Kalkhof S, Ihling C, Kolbel K, Ashery U, Rhee J, Brose N, Sinz A, Jahn O (2012) Nonconserved  $Ca_2+$ /calmodulin binding sites in Munc13s differentially control synaptic short-term plasticity. *Mol Cell Biol* 32(22):4628–4641. <https://doi.org/10.1128/Mcb.00933-12>
- Liu F, Rijkers DT, Post H, Heck AJ (2015) Proteome-wide profiling of protein assemblies by cross-linking mass spectrometry. *Nat Methods* 12(12):1179–1184. <https://doi.org/10.1038/nmeth.3603>
- Liu F, Lossl P, Scheltema R, Viner R, Heck AJR (2017) Optimized fragmentation schemes and data analysis strategies for proteome-wide cross-link identification. *Nat Commun* 8:15473. <https://doi.org/10.1038/ncomms15473>
- Lössl P, Sinz A (2016) Combining amine-reactive cross-linkers and photo-reactive amino acids for 3D-structure analysis of proteins and protein complexes. *Methods Mol Biol* 1394:109–127. [https://doi.org/10.1007/978-1-4939-3341-9\\_9](https://doi.org/10.1007/978-1-4939-3341-9_9)
- Lössl P, Kölbl K, Tänzler D, Nannemann D, Ihling CH, Keller MV, Schneider M, Zaucke F, Meiler J, Sinz A (2014) Analysis of Nidogen-1/laminin gamma1 interaction by cross-linking, mass spectrometry, and computational modeling reveals multiple binding modes. *PLoS One* 9(11):e112886. <https://doi.org/10.1371/journal.pone.0112886>
- Maadi H, Nami B, Wang Z (2017) Dimerization assessment of epithelial growth factor family of receptor tyrosine kinases by using cross-linking reagent. *Methods Mol Biol* 1652:101–108. [https://doi.org/10.1007/978-1-4939-7219-7\\_6](https://doi.org/10.1007/978-1-4939-7219-7_6)
- Mädler S, Bich C, Touboul D, Zenobi R (2009) Chemical cross-linking with NHS esters: a systematic study on amino acid reactivities. *J Mass Spectrom* 44(5):694–706. <https://doi.org/10.1002/jms.1544>
- Maupetit J, Derreumaux P, Tuffery P (2009) PEP-FOLD: an online resource for de novo peptide structure prediction. *Nucleic acids research* 37 (web server issue):W498–503. <https://doi.org/10.1093/nar/gkp323>
- McIlwain S, Tamura K, Kertesz-Farkas A, Grant CE, Diamant B, Frewen B, Howbert JJ, Hoopmann MR, Kall L, Eng JK, MacCoss MJ, Noble WS (2014) Crux: rapid open source protein tandem mass spectrometry analysis. *J Proteome Res* 13(10):4488–4491. <https://doi.org/10.1021/pr500741y>
- Merkley ED, Rysavy S, Kahraman A, Hafen RP, Daggett V, Adkins JN (2014) Distance restraints from crosslinking mass spectrometry: mining a molecular dynamics simulation database to evaluate lysine-lysine distances. *Prot Sci* 23(6):747–759. <https://doi.org/10.1002/pro.2458>
- Müller DR, Schindler P, Towbin H, Wirth U, Voshol H, Hoving S, Steinmetz MO (2001) Isotope-tagged cross-linking reagents. A new tool in mass spectrometric protein interaction analysis. *Anal Chem* 73(9):1927–1934
- Müller MQ, Schafer M, Dreier F, Ihling CH, Sinz A (2010) Cleavable cross-linker for protein structure analysis: reliable identification of cross-linking products by tandem MS. *Anal Chem* 82(16):6958–6968. <https://doi.org/10.1021/ac101241t>
- Nielsen T, Thaysen-Andersen M, Larsen N, Jorgensen FS, Houen G, Hojrup P (2007) Determination of protein conformation by isotopically labelled cross-linking and dedicated software: application to the chaperone, calreticulin. *Int J Mass Spectrom* 268(2–3):217–226. <https://doi.org/10.1016/j.jims.2007.06.019>

- Novak P, Kruppa GH (2008) Intra-molecular cross-linking of acidic residues for protein structure studies. *Eur J Mass Spectrom* 14(6):355–365. <https://doi.org/10.1255/ejms.963>
- Nury C, Redeker V, Dautrey S, Romieu A, van der Rest G, Renard PY, Melki R, Chamot-Rooke J (2015) A novel bio-orthogonal cross-linker for improved protein/protein interaction analysis. *Anal Chem* 87(3):1853–1860. <https://doi.org/10.1021/ac503892c>
- Operana TN, Tukey RH (2007) Oligomerization of the UDP-glucuronosyltransferase 1A proteins: homo- and heterodimerization analysis by fluorescence resonance energy transfer and co-immunoprecipitation. *J Biol Chem* 282(7):4821–4829. <https://doi.org/10.1074/jbc.M609417200>
- Panchaud A, Singh P, Shaffer SA, Goodlett DR (2010) xComb: a cross-linked peptide database approach to protein-protein interaction analysis. *J Proteome Res* 9(5):2508–2515. <https://doi.org/10.1021/pr9011816>
- Petrochenko EV, Serpa JJ, Hardie DB, Berjanskii M, Suriyamongkol BP, Wishart DS, Borchers CH (2012) Use of proteinase K nonspecific digestion for selective and comprehensive identification of interpeptide cross-links: application to prion proteins. *Mol Cell Proteom* 11(7):M111 013524. <https://doi.org/10.1074/mcp.M111.013524>
- Petrochenko EV, Makepeace KA, Borchers CH (2014) DXMSMS match program for automated analysis of LC-MS/MS data obtained using isotopically coded CID-cleavable cross-linking reagents. *Curr Prot Bioinform* 48:8.18.11–19. doi:<https://doi.org/10.1002/0471250953.bi0818s48>
- Piotrowski C, Ihling CH, Sinz A (2015) Extending the cross-linking/mass spectrometry strategy: facile incorporation of photo-activatable amino acids into the model protein calmodulin in *Escherichia coli* cells. *Methods* 89:121–127. <https://doi.org/10.1016/j.ymeth.2015.02.012>
- Politis A, Stengel F, Hall Z, Hernandez H, Leitner A, Walzthoeni T, Robinson CV, Aebersold R (2014) A mass spectrometry-based hybrid method for structural modeling of protein complexes. *Nat Methods* 11(4):403–406. <https://doi.org/10.1038/nmeth.2841>
- Puig O, Caspary F, Rigaut G, Rutz B, Bouveret E, Bragado-Nilsson E, Wilm M, Seraphin B (2001) The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* 24(3):218–229. <https://doi.org/10.1006/meth.2001.1183>
- Rampl E, Stranzl T, Orban-Nemeth Z, Hollenstein DM, Hudcz O, Schloegelhofer P, Mechtler K (2015) Comprehensive cross-linking mass spectrometry reveals parallel orientation and flexible conformations of plant HOP2-MND1. *J Proteome Res* 14(12):5048–5062. <https://doi.org/10.1021/acs.jproteome.5b00903>
- Rappsilber J (2011) The beginning of a beautiful friendship: cross-linking/mass spectrometry and modelling of proteins and multi-protein complexes. *J Struct Biol* 173(3):530–540. <https://doi.org/10.1016/j.jsb.2010.10.014>
- Rasmussen MI, Refsgaard JC, Peng L, Houen G, Hojrup P (2011) CrossWork: software-assisted identification of cross-linked peptides. *J Proteome* 74(10):1871–1883. <https://doi.org/10.1016/j.jprot.2011.04.019>
- Rinner O, Seebacher J, Walzthoeni T, Mueller LN, Beck M, Schmidt A, Mueller M, Aebersold R (2008) Identification of cross-linked peptides from large sequence databases. *Nat Methods* 5(4):315–318. <https://doi.org/10.1038/nmeth.1192>
- Ryu Y, Schultz PG (2006) Efficient incorporation of unnatural amino acids into proteins in *Escherichia coli*. *Nat Methods* 3(4):263–265 nmeth864 [pii] <https://doi.org/10.1038/nmeth864>
- Sarpe V, Rafiei A, Hepburn M, Ostan N, Schryvers AB, Schriemer DC (2016) High sensitivity crosslink detection coupled with integrative structure modeling in the mass spec studio. *Mol Cell Proteomics* 15(9):3071–3080. <https://doi.org/10.1074/mcp.O116.058685>
- Schilling B, Row RH, Gibson BW, Guo X, Young MM (2003) MS2Assign, automated assignment and nomenclature of tandem mass spectra of chemically crosslinked peptides. *J Am Soc Mass Spectrom* 14(8):834–850. [https://doi.org/10.1016/S1044-0305\(03\)00327-1](https://doi.org/10.1016/S1044-0305(03)00327-1)
- Schmidt R, Sinz A (2017) Improved single-step enrichment methods of cross-linked products for protein structure analysis and protein interaction mapping. *Anal Bioanal Chem* 409(9):2393–2400. <https://doi.org/10.1007/s00216-017-0185-1>

- Schmidt A, Kalkhof S, Ihling C, Cooper DM, Sinz A (2005) Mapping protein interfaces by chemical cross-linking and Fourier transform ion cyclotron resonance mass spectrometry: application to a calmodulin/adenylyl cyclase 8 peptide complex. *Eur J Mass Spectrom* 11(5):525–534. <https://doi.org/10.1255/ejms.748>
- Schwarz R, Tanzler D, Ihling CH, Sinz A (2016) Monitoring solution structures of peroxisome proliferator-activated receptor beta/delta upon ligand binding. *PLoS One* 11(3):e0151412. <https://doi.org/10.1371/journal.pone.0151412>
- Schweppe DK, Chavez JD, Lee CF, Caudal A, Kruse SE, Stuppard R, Marcinek DJ, Shadel GS, Tian R, Bruce JE (2017) Mitochondrial protein interactome elucidated by chemical cross-linking mass spectrometry. *Proc Natl Acad Sci U S A* 114(7):1732–1737. <https://doi.org/10.1073/pnas.1617220114>
- Sinz A (2006) Chemical cross-linking and mass spectrometry to map three-dimensional protein structures and protein-protein interactions. *Mass Spectrom Rev* 25(4):663–682. <https://doi.org/10.1002/mas.20082>
- Sinz A (2014) The advancement of chemical cross-linking and mass spectrometry for structural proteomics: from single proteins to protein interaction networks. *Expert Rev Proteomics* 11(6):733–743. <https://doi.org/10.1586/14789450.2014.960852>
- Sinz A (2017) Divide and conquer: cleavable cross-linkers to study protein conformation and protein-protein interactions. *Anal Bioanal Chem* 409(1):33–44. <https://doi.org/10.1007/s00216-016-9941-x>
- Soderberg CA, Lambert W, Kjellstrom S, Wiegandt A, Wulff RP, Mansson C, Rutsdottir G, Emanuelsson C (2012) Detection of crosslinks within and between proteins by LC-MALDI-TOF/TOF and the software FINDX to reduce the MSMS-data to acquire for validation. *PLoS One* 7(6):e38927. <https://doi.org/10.1371/journal.pone.0038927>
- Speers AE, Cravatt BF (2005) A tandem orthogonal proteolysis strategy for high-content chemical proteomics. *J Am Chem Soc* 127(28):10018–10019. <https://doi.org/10.1021/ja0532842>
- Suchanek M, Radzikowska A, Thiele C (2005) Photo-leucine and photo-methionine allow identification of protein-protein interactions in living cells. *Nat Methods* 2(4):261–267. <https://doi.org/10.1038/nmeth752>
- Tan D, Li Q, Zhang MJ, Liu C, Ma C, Zhang P, Ding YH, Fan SB, Tao L, Yang B, Li X, Ma S, Liu J, Feng B, Liu X, Wang HW, He SM, Gao N, Ye K, Dong MQ, Lei X (2016) Trifunctional cross-linker for mapping protein-protein interaction networks and comparing protein conformational states. *eLife* 5. <https://doi.org/10.7554/eLife.12509>
- Tang X, Bruce JE (2010) A new cross-linking strategy: protein interaction reporter (PIR) technology for protein-protein interaction studies. *Mol BioSyst* 6(6):939–947. <https://doi.org/10.1039/b920876c>
- Tang Y, Chen Y, Lichti CF, Hall RA, Raney KD, Jennings SF (2005) CLPM: a cross-linked peptide mapping algorithm for mass spectrometric analysis. *BMC bioinformatics* 6 Suppl 2:S9. <https://doi.org/10.1186/1471-2105-6-S2-S9>
- Tinnefeld V, Venne AS, Sickmann A, Zahedi RP (2017) Enrichment of cross-linked peptides using charge-based fractional diagonal chromatography (ChaFRADIC). *J Proteome Res* 16:459–469. <https://doi.org/10.1021/acs.jproteome.6b00587>
- Walker-Gray R, Stengel F, Gold MG (2017) Mechanisms for restraining cAMP-dependent protein kinase revealed by subunit quantitation and cross-linking approaches. *Proc Natl Acad Sci U S A* 114(39):10414–10419. <https://doi.org/10.1073/pnas.1701782114>
- Walzthoeni T, Leitner A, Stengel F, Aebersold R (2013) Mass spectrometry supported determination of protein complex structure. *Curr Opin Struct Biol* 23(2):252–260. <https://doi.org/10.1016/j.sbi.2013.02.008>
- Walzthoeni T, Joachimiak LA, Rosenberger G, Rost HL, Malmstrom L, Leitner A, Frydman J, Aebersold R (2015) xTract: software for characterizing conformational changes of protein complexes by quantitative cross-linking mass spectrometry. *Nat Methods* 12(12):1185–1190. <https://doi.org/10.1038/nmeth.3631>

- Weerapana E, Speers AE, Cravatt BF (2007) Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)—a general method for mapping sites of probe modification in proteomes. *Nat Protoc* 2(6):1414–1425. <https://doi.org/10.1038/nprot.2007.194>
- Weisbrod CR, Chavez JD, Eng JK, Yang L, Zheng C, Bruce JE (2013) In vivo protein interaction network identified with a novel real-time cross-linked peptide identification strategy. *J Proteome Res* 12(4):1569–1579. <https://doi.org/10.1021/pr3011638>
- Weisz DA, Liu H, Zhang H, Thangapandian S, Tajkhorshid E, Gross ML, Pakrasi HB (2017) Mass spectrometry-based cross-linking study shows that the Psb28 protein binds to cytochrome b559 in photosystem II. *Proc Natl Acad Sci U S A* 114(9):2224–2229. <https://doi.org/10.1073/pnas.1620360114>
- Wittelsberger A, Thomas BE, Mierke DF, Rosenblatt M (2006) Methionine acts as a “magnet” in photoaffinity crosslinking experiments. *Febs Lett* 580(7):1872–1876. <https://doi.org/10.1016/j.febslet.2006.02.050>
- Xu H, Zhang L, Freitas MA (2008) Identification and characterization of disulfide bonds in proteins and peptides from tandem MS data by use of the MassMatrix MS/MS search engine. *J Proteome Res* 7(1):138–144. <https://doi.org/10.1021/pr070363z>
- Yang B, Wu YJ, Zhu M, Fan SB, Lin J, Zhang K, Li S, Chi H, Li YX, Chen HF, Luo SK, Ding YH, Wang LH, Hao Z, Xiu LY, Chen S, Ye K, He SM, Dong MQ (2012) Identification of cross-linked peptides from complex samples. *Nat Methods* 9(9):904–906. <https://doi.org/10.1038/nmeth.2099>
- Yang Y, Song H, Chen PR (2016a) Genetically encoded photocrosslinkers for identifying and mapping protein-protein interactions in living cells. *IUBMB Life* 68(11):879–886. <https://doi.org/10.1002/iub.1560>
- Yang Y, Song H, He D, Zhang S, Dai S, Lin S, Meng R, Wang C, Chen PR (2016b) Genetically encoded protein photocrosslinker with a transferable mass spectrometry-identifiable label. *Nat Commun* 7:12299. <https://doi.org/10.1038/ncomms12299>
- Yilmaz S, Drepper F, Hulstaert N, Cernic M, Gevaert K, Economou A, Warscheid B, Martens L, Vandermarliere E (2016) Xilmass: a new approach toward the identification of cross-linked peptides. *Anal Chem* 88(20):9949–9957. <https://doi.org/10.1021/acs.analchem.6b01585>
- Young MM, Tang N, Hempel JC, Oshiro CM, Taylor EW, Kuntz ID, Gibson BW, Dollinger G (2000) High throughput protein fold identification by using experimental constraints derived from intramolecular cross-links and mass spectrometry. *Proc Natl Acad Sci U S A* 97(11):5802–5806. <https://doi.org/10.1073/pnas.090099097>
- Yu F, Li N, Yu W (2016) ECL: an exhaustive search tool for the identification of cross-linked peptides using whole database. *BMC Bioinform* 17(1):217. <https://doi.org/10.1186/s12859-016-1073-y>
- Yu F, Li N, Yu W (2017) Exhaustively identifying cross-linked peptides with a linear computational complexity. *J Proteome Res* 16(10):3942–3952. <https://doi.org/10.1021/acs.jproteome.7b00338>
- Zhang Y (2009) I-TASSER: fully automated protein structure prediction in CASP8. *Proteins* 77(Suppl 9):100–113. <https://doi.org/10.1002/prot.22588>
- Zheng C, Weisbrod CR, Chavez JD, Eng JK, Sharma V, Wu X, Bruce JE (2013) XLink-DB: database and software tools for storing and visualizing protein interaction topology data. *J Proteome Res* 12(4):1989–1995. <https://doi.org/10.1021/pr301162j>
- Ziemanowicz DS, Bomgarden R, Etienne C, Schriemer DC (2017) Amino acid insertion frequencies arising from photoproducts generated using aliphatic Diazirines. *J Am Soc Mass Spectrom* 28:2011–2021. <https://doi.org/10.1007/s13361-017-1730-z>