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Photo-Assisted Peptide Enrichment in Protein Complex Cross-Linking Analysis of a Model Homodimeric Protein Using Mass Spectrometry

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Abstract

Mass spectrometry analysis of cross-linked peptides can be used to probe protein contact sites in macromolecular complexes. We have developed a photo-cleavable cross-linker that enhances peptide enrichment, improving the signal-to-noise ratio of the cross-linked peptides in mass spectrometry analysis. This cross-linker utilizes nitro-benzyl alcohol group that can be cleaved by UV irradiation and is stable during the multiple washing steps used for peptide enrichment. The enrichment method utilizes a cross-linker that aids in eliminating contamination resulting from protein based retrieval systems, and thus, facilitates the identification of cross-linked peptides. Homodimeric pilM protein from *Pseudomonas aeruginosa* 2192 (pilM) was investigated to test the specificity and experimental conditions. As predicted, the known pair of lysine side chains within 14Å was cross-linked. An unexpected cross-link involving the protein's amino terminus was also detected. This is consistent with the predicted mobility of the amino terminus that may bring the amino groups within 19Å of one another in solution. These technical improvements allow this method to be used for investigating protein-protein interactions in complex biological samples.

Keywords

cross-link; enrichment; photo-cleavable; transient protein complex

The cross-linkers used in bioconjugate analysis are as varied as their proposed applications[1,2]. Whether tagging proteins to make them fluorescent, labeling molecules with biospecific ligands or cross-linking two or more substances to create uniquely active conjugates, an increasing number of bioconjugate reagents is becoming available[3,4]. The selection of cross-linkers novel reactive groups has also grown along with the variety of their design for mass spectrometric proteomics studies[5,6]. Photo-cleavable cross-linkers can be used for specific modifications, and are receiving significant attention in biotechnology applications[7,8]. The use of a photo-cleavable cross-linker provides a convenient and clean way to attach and/or detach biological molecules from a substrate[9,10,11]. Photo-cleavable biotin derivatives were designed over a decade ago as a

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versatile approach for the isolation of biomolecules[12,13]. A bead affinity chromatography system was also developed based on a photo-cleavable cross-linker [14]. Micro affinity purification has proven to be a successful method for the identification of specific target proteins in a protein mixture. Recently, Specht described using homo-bifunctional photo-cleavable cross-linkers to photo-regulate the cleavage[15]. These chemical probes were able to react with two equivalents of cysteine, and efficiently photo-release them with near-UV irradiation[16].

To achieve enrichment, affinity purifications based on chemically modified beads are widely used. Solid-phase chemical reaction based modification is clean and does not require high temperature or harsh conditions for synthesis[17]. A wide range of reagents are now commercially available. However, novel linkers having a specific functional group, such as an affinity or isotopic labeled tag, still need to be synthesized. These would circumvent the present limitations of current linkers and allow for development of new reagents to extend the use of cross-linkers.

To design a photo-cleavable cross-linker with desired functions, the cross-linkers must first be stable to visible light, solvents and buffers so that researchers have sufficient time to manipulate the linker and complete the modification. Second, the active groups in cross-linkers must be reactive to common substrates of the targeted biomolecules. The targeted biomolecule must also be stable enough for multi-step immobilization without losing their original activities. Third, the cross-linker should be cleavable at wavelengths longer than 300nm in order to get minimal damage to biomolecules during UV irradiation. Shorter wavelength light with higher energy can damage biomolecules[18]. Finally, the quantum yield of the photolytic reaction should be high enough so that photolysis is accomplished within a short time period (e.g. 10 minutes). The acceptable quantum yield value is between 0.008 and 0.3[19].

Previously, we reported on the development of a photo-assisted selective retrieval method to enrich cross-linked peptides for mass spectrometry analysis[20]. A photo-cleavable cross-linker, succinimidyl 5-(3-iodo-propoxy)-2-nitro-benzyl carbonic ester (SINB) was synthesized and its application in cross-linked peptide enrichment was demonstrated. However, as a carbonate ester, SINB is not stable in water during sample preparation, which affects the efficiency of enrichment. In order to improve the stability of the photo-cleavable cross-linker in aqueous solutions we developed and synthesized a succinic acid α -nitrobenzyl ester (succinimidyl 5-(3-iodo-propoxy)-2-nitro-benzyl succinic ester, SSIN (Fig. 1.). An iodo group (iodo-propanyl) is attached to the phenyl through an ether bond, which, after the succinimidyl ester reacts with the amino group, can anchor the thiol group of cross-linked peptides to the beads (Fig. 1). The succinic acid α -nitrobenzyl ester bond is robust enough during bead modification and multiple washing steps, and is cleaved only after UV irradiation, thus permitting selective retrieval of the linked peptides.

To characterize the photochemical properties, SSIN was irradiated under UV 355nm using two-second intervals. The quantum efficiency was 12%, with an isosbestic point at 328nm (See Supporting Material Fig.S2). To confirm the functionality of SSIN in the selective retrieval of cross-linked peptides, a mixture of two peptides (Xenopsin-related peptide I, FHPKRPWIL, monoisotopic m/z 1193.69 and a substrate of Protein Tyrosine Kinase, RRLIEDNEYTARG, monoisotopic m/z 1592.81) was cross-linked with SAMS and then enriched with SINB and SSIN modified magnetic beads, respectively. SSIN immobilized beads remain intact even after overnight soaking in PBS. These improvements allowed us to obtain a dramatic enrichment of modified Xenopsin-related peptide at m/z 1383.72. The detailed results and discussion are shown in figure S3 in the supporting materials.

With this promising result, we further investigated our system using a 27.5 kDa homodimeric pilM protein (Protein Data Bank code: 3HG9) as a model. The structure of pilM was confirmed by X-ray crystallography to be a homodimer. Each monomer has 131 residues (Fig. 2). The protein pilM was chosen from the Protein Data Bank because of its small size and because it possesses only 2 lysines per chain thus, rendering the cross-link assignments from MS/MS data straightforward. Additionally, pilM forms a weak dimeric complex and easily separates into monomers. Therefore, it serves as a good model system to mimic *in vivo* systems and will provide valuable information to further our understanding of the mechanism involved during cross-linking of other transient protein complexes. Formation of transient protein complexes is a frequent cellular event and a characteristic feature of signaling pathways important in carcinogenesis and other cell processes. Finally, the pilM complex was chosen because of its advantageous arrangement of Lys residues, *i.e.* there is an interchain contact clearly within the span of our cross-linker (K_A123-K_B123 : 13.98 Å) and at least one Lys-Lys distance (K_A53-K_B53 : 44.29 Å), which greatly exceeds the distance (19.5 Å). This arrangement allows us to monitor specific and non-specific cross-links within the same system. Since there is no cysteine in pilM, the reduction and alkylation steps were omitted during digestion. Modified lysines that cannot be enzymatically digested may produce cross-linked peptides over 6000 Da, thus being difficult for MS/MS sequencing. Therefore, we chose two enzymes, trypsin and Glu-C for digestion. The enzymatic digestion resulted in proteolytic peptides that provided 96.2% coverage of the protein sequence (Fig 2a). Detailed results follow below.

Although the crystal structure of pilM is a homodimer (Fig. 2b), the majority of protein in solution exists as monomers, as observed by our MALDI-TOF MS and size exclusion chromatography results (Data not shown). Therefore, it was a challenging task to generate cross-links in this weak complex. We tried different molar ratios between lysine and SAMS (1:4 to 1:12) to maximize the amount of dimers and cross-links of K_A123-K_B123 . We also carried out the cross-linking reaction under different temperatures between 4 and 37 °C. We have observed that the amount of cross-linker generally correlates with the amount of observed dimers. However, when too much cross-linker was used (at mol:mol > 1:10), an increase in non-specific cross-links were generated. The optimal conditions are a Lys:SAMS ratio of 1:8 (mol:mol) at a temperature of 4°C.

When untreated pilM was digested, we observed 12 major proteolytic peptides (Table 1). Since lysine 53 is followed by a proline the peptide bond is not cleavable by trypsin, resulting in the peptide SALGLPAWFRKPVR (m/z 1597.87) as observed. The C-terminal peptide (EGHHHHHH, m/z 1027.44) was also observed. After cross-linking, K53 becomes dead-end cross-linked and this modified peptide is found at m/z 1771.93 with a very weak intensity. The C-terminal peptide, EGHHHHHH is not observed after cross-linking since the K-E peptide bond is not cleavable after lysine is modified. Instead, a stronger signal intensity of the GHHHHHHH (m/z 898.38) peptide is observed as a Glu-C digested peptide. Before enrichment, the cross-linked peptides were barely detected due to their low intensity. As shown in Figure 3a, the dominant observable peptides were GHHHHHHH, SALGLPAWFR, TLARSLLYR, RLGATAIALPAPIPE, YAHANPGFSGSPADSALGLPAWFR and LQGYIAAGTSYAFIASPPAGLAAAVDTGTE (m/z 898.38, 1117.62, 1205.74, 1489.87, 2489.21, and 2883.45, respectively).

The absolute ion counts of the cross-linked peptides before enrichment are: lysine cross-linked peptide GAVVAVK(GAVVAVKE)E (m/z 1669.96, 38), dead-end labeled peptide SALGLPAWFRK(Linker)PVR (m/z 1771.93, 30), N-terminal cross-linked peptide NH₂-M(NH₂-MSLTSSAE)SLTSSAEAE (m/z 2118.86, 84), and lysine and N-terminal cross-linked peptide LGATAIALPAPIPEGAVVAVK(MSLTSSAEAEVD)E (m/z 3594.82, 84).

After enrichment, the cross-linked peptides were observed as dominant peaks (Fig 3b) with 16 Dalton increase, which is a consequence of the addition of CH₄ due to the enrichment procedure [18]. As a result, the absolute ion counts of the cross-linked peptides are: m/z 1715.92 (505), m/z 1787.95 (136), m/z 2134.89 (86), m/z 3610.85 (189). The percent improvements in intensity are listed in the table and the maximum yield could be as high as 1558%. The sequences of the peptides were confirmed by TOF/TOF. The major product ions observed in the MS/MS spectrum of peptide with m/z 1715.93 (Fig. 3c) are $b_{2\alpha}^+$, $b_{3\alpha}^+$, $b_{6\alpha}^+$, $y_{2\alpha}^+$, $y_{3\alpha}^+$, $y_{5\alpha}^+$, $y_{6\alpha}^+$, $y_{7\alpha}^+$, and internal fragmentation ions such as $[b_{7\alpha}y_{3\alpha}y_{2\beta}-H_2O]^+$, $[VK(KE)-H_2O]^+$, (m/z 657.32); $[b_{7\alpha}y_{3\alpha}y_{2\beta}]^+$, $[VK(KE)]^+$, (m/z 675.33); $[b_{7\alpha}y_{6\alpha}y_{2\beta}]^+$, $[VVAVK(KE)]^+$, (m/z 944.52); $[y_{5\alpha}b_{7\beta}y_{4\beta}]^+$, $[VAVK(AVK)E]^+$, (m/z 997.51); $[b_{7\alpha}b_{7\beta}y_{4\beta}]^+$ $[GAVVAVK(AVK)]^+$, (m/z 1095.64). When the two cross-linked peptides are identical, the labeled α or β product ions can apply to either one. In all other cases, the product ions of the longer cross-linked peptide are noted as α and the shorter one as β . The nomenclature used in the MS/MS spectra is in accordance with that suggested by Schilling and coauthors [21]. When the lysine residues are cross-linked, the total peptide mass of the other cross-linked peptide is included in the calculated mass. Figure 3d is the MS/MS spectrum of the peptide with m/z 3610.85. MS/MS spectra of peptides with m/z 1787.95 and m/z 2134.89 are provided in Figure S4 in the supporting materials.

The surface of the bead contains many phenyl groups from polystyrene and from the nitro-benzyl group of the photo-cleavable spacer, thus increasing the potential for enrichment of non-cross-linked peptides by non-specific absorption. The major peptides shown in Fig. 3a, such as SALGLPAWFR (m/z 1117.62), TLARSLLLYR (m/z 1205.74), RLGATAIALPAPIPE (m/z 1489.87), and YAHANPGFSGSPADSALGLPAWFR (m/z 2489.21), all contain tyrosine and/or phenylalanine residues and thus, are absorbed to the beads non-specifically. To efficiently remove these physically absorbed peptides, the beads were soaked in buffer (0.01% Tween20) with shaking at 37°C for one hour during each washing step. These additional wash procedures allowed us to more specifically enrich for cross-linked peptides (Fig. 3b). This method adjustment has proven to improve the signal-to-noise of cross-linked peptides of interest and minimize non-specific peptide absorption.

Another important note is to properly control the amount of cross-linking reagent. An insufficient amount of cross-linker will not generate enough cross-links. When the amount of SAMS is greater than ten times the mole amount of lysines present in the protein, all dead end cross-links of lysines and N-terminal are observed.

From the crystal structure of the pilM dimer (Fig. 2b), we can easily see that K_A123 and K_B123 are the closest lysine pair. This cross-link was observed as m/z ion 1715.92 (Fig. 3c). Lysine 53 is only observed as a dead-end labeled cross-linked peptide with m/z at 1787.95. To our surprise, the N-terminal showed certain activity and can be cross-linked with K123. The distance of the N-terminal to K123 is 33.2 Å and therefore, not likely to be linked under this condition. Probably the N-terminus could swing in solution and was caught by the linker to generate a “false” dimer during cross-linking. To minimize this cross-link, the experiment was carried out at 4°C, we observed that the amount of N-terminal to K123 cross-links was reduced. This suggests that lower temperature reduces the mobility of the N-terminal, and hence avoids most of the cross-links of N-terminal to other primary amines.

In conclusion, we described the design and synthesis of a novel photo-cleavable cross-linker to improve the efficiency of cross-linked peptide enrichment technology. The cross-linked peptides were anchored to beads with a photo-cleavable spacer and then enriched with photo-irradiation. The signal of MS/MS spectra was improved allowing for unambiguous sequence assignment. The method described here provides an alternative platform to the single, commercially available biotin based affinity purification method to enrich cross-

linked peptides. As a non-protein based enrichment strategy, this method can be applied to examine and map protein-protein interaction in complex samples and in living cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NHS	<i>N</i> -hydroxysuccinimide
SAMS	2-acetylsulfanyl-succinic acid bis-succinimidyl ester
SINB	succinimidyl 5-(3-iodo-propoxy)-2-nitro-benzyl carbonic ester
SSIN	succinimidyl 5-(3-iodo-propoxy)-2-nitro-benzyl succinic ester
pilM	Homodimeric pilM protein

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Figure 1. Schema of enrichment of cross-linked peptides. Magnetic beads were modified by SSIN: (Step A) magnetic beads are modified by SSIN; (Step B) SSIN modified beads are incubated with the proteolytic peptides; (Step C) after removal of the non-specifically bound peptides, UV irradiation is applied to release the cross-linked peptides for tandem mass spectrometry analysis.

Figure 2. Amino acid sequence (a) and crystal structure of the 27.5 kDa homodimeric pilM protein(b). The observed proteolytic peptide coverage map of pilM is underlined(a). Lysines and N-terminals are highlighted as spacefilled balls(b).

Figure 3.

MALDI-TOF/TOF MS spectra of cross-linked peptides. Tryptic/Glu-C digested peptides before selective retrieval (a) and after selective retrieval of peptides using the SSIN modified beads (b). The cross-linked peptide absolute counts before and after enrichment are: m/z 1715.92(38, 505), m/z 1787.95(30, 136), m/z 2134.89(84, 86), m/z 3610.85(84, 189). These are Lysine cross-linked peptide GAVVAVK(GAVVAVKE)E (m/z 1715.92), dead-end labeled peptide SALGLPAWFRK(Linker)PVR(m/z 1787.95), N-terminal cross-linked peptide NH₂-M(NH₂-MSLTSSAE)SLTSSAELAE(m/z 2134.89), and Lysine and N-terminal cross-linked peptide LGATAIALPAIPEGAVVAVK(MSLTSSAELAEVD)E (m/z 3610.85). Black triangles indicate the enriched cross-linked peptides. MS/MS of precursor 1715.92(c) and of precursor m/z 3610.85(d). The observed monoisotopic mass error is less than 10ppm from the predicted mass for most ions. The product ions of the longer cross-linked peptide are noted as α and the shorter one as β . When the lysine residues are cross-linked, the total peptide mass of the other cross-linked peptide is included in the calculated mass.

Table 1

Comparison of observed MS/MS peaks of digested peptides detected in three experiments: (1) non-enriched/non-cross-linked("untreated"), (2) cross-linked and (3) cross-linked and enriched samples of pIIM.

Predicted monoisotopic protonated ions(<i>m/z</i>)	Sequence position	Sequence of digested peptides	Absolute counts(Signal to noise ratio)			
			Untreated pIIM	Cross-linked pIIM	Enriched pIIM	Improved S/N ratio
999.36	1~8 ¹ Label	MSLTSSAE ¹	*	*	*	
1138.53	1~11	MSLTSSAE LAE	321(332)	*	*	
2539.34	1~23	MSLTSSAE LAE VDTLARSLLLYR	955(949)	517(506)	21(24)	
1312.52	1~11 ¹ Label	MSLTSSAE LAE	*	*	*	
2432.04	1~11 ¹ ~11	MSLTSSAE LAE ¹ MSLTSSAE LAE	*	*	*	
2118.88	1~8 ¹ ~11	MSLTSSAE LAE ¹ MSLTSSAE	*	84(71)	*	
2134.92	1~8 ¹ ~11	MSLTSSAE LAE ¹ MSLTSSAE	*	*	86(102)	143%
1805.71	1~8 ¹ ~8	MSLTSSAE ¹ MSLTSSAE	*	*	*	
2289.29	9~28	LAE VDTLARSLLLYRSRLAE	391(398)	881(826)	*	
1205.74	14~23	TLARSLLLYR	*	1832(1791)	*	
3133.62	14~42	TLARSLLLYRSRLAEYAHANPGFSGSPAD	*	114(109)	7(13)	
2802.36	26~52	LAEYAHANPGFSGSPADSALGLPAWFR	*	38(33)	*	
3456.68	26~56 ¹ Label	LAEYAHANPGFSGSPADSALGLPAWFRKPVR ¹	*	*	*	
1389.60	29~42	YAHANPGFSGSPAD	522(571)	*	*	
2471.20	29~52	YAHANPGFSGSPADSALGLPAWFR	281(277)	2439(2381)	*	
2489.20	29~52	YAHANPGFSGSPADSALGLPAWFR	5672(5711)	6179(6022)	45(51)	
1117.62	43~52	SALGLPAWFR	3803(3820)	1999(1891)	9(14)	
1597.93	43~56	SALGLPAWFRKPVR	1529(1571)	*	*	
1771.93	43~56 ¹ Label	SALGLPAWFRKPVR ¹	*	30(31)	*	
1787.97	43~56 ¹ Label	SALGLPAWFRKPVR ¹	*	*	136(162)	522%
1153.65	53~56 ¹ 53~56	KPVR ¹ KPVR	*	*	*	

Predicted monoisotopic protonated ions(<i>m/z</i>)	Sequence position	Sequence of digested peptides	Absolute counts(Signal to noise ratio)			
			Untreated p1M	Cross-linked p1M	Enriched p1M	Improved S/N ratio
1479.68	53~56 ¹ ~8	KPVR ¹ MSLTSSAE	*	*	*	*
1792.84	53~56 ¹ ~8	KPVR ¹ MSLTSSAE	*	*	*	*
2741.52	53~56 ¹ 103~124	KPVR ¹ LGATAIALPAPIEGAVVAVKE	*	*	*	*
1426.77	53~56 ¹ 117~124	KPVR ¹ GAVVAVKE	*	*	*	*
2495.28	57~82	LQGYIAAGTSYAFIASPPAGLAAAVD	311(344)	*	*	*
2883.44	57~86	LQGYIAAGTSYAFIASPPAGLAAAVDTGTE	*	46(41)	*	*
1669.95	87~101	SDLVGVRRNGQLVTR	*	99(92)	*	*
1099.64	94~102	NGQLVTRR	*	106(101)	*	*
1489.87	102~116	LGATAIALPAPIE	5574(5942)	1618(1622)	36(39)	
3594.82	103~124 ¹ ~13	LGATAIALPAPIEGAVVAVKE ¹ MSLTSSAE ¹ AEVD	*	84(77)	*	
3610.86	103~124 ¹ ~13	LGATAIALPAPIEGAVVAVKE ¹ MSLTSSAE ¹ AEVD	*	*	189(202)	262%
2065.96	117~124 ¹ ~11	GAVVAVKE ¹ MSLTSSAE ¹ AE	*	*	*	
1752.80	117~124 ¹ ~8	GAVVAVKE ¹ MSLTSSAE	*	*	*	
1699.89	117~124 ¹ 117~124	GAVVAVKE ¹ GAVVAVKE	*	38(36)	*	
1715.93	117~124 ¹ 117~124	GAVVAVKE ¹ GAVVAVKE	*	*	505(561)	1558%
2945.34	117~131 ¹ ~11	GAVVAVKEGHHHHHH ¹ MSLTSSAE ¹ AE	*	*	*	
946.40	117~124 ¹ Label	GAVVAVKE ¹	*	*	*	
1027.43	124~131	EGHHHHHH	81(92)	*	*	
898.39	125~131	GHHHHHH	302(362)	1178(1169)	*	

The carat(^)represents the cross-link.

The tilde(~) represents the peptides within the sequence.

An asterisk(*) represents that the predicted ion is not observed;

"Label" in sequence position column represents the dead-end cross-link.