Influence of cross-linker polarity on selectivity towards lysine side chains

Jan Fiala[1,2], Zdeněk Kukačka[1] and Petr Novák[1,2,*]

[1] Institute of Microbiology of the Czech Academy of Sciences, Vestec, Czech Republic
[2] Department of Biochemistry, Faculty of Science, Charles University, Prague, Czech Republic

Correspondence
P. Novák, Institute of Microbiology of the Czech Academy of Sciences, Průmyslová 595, 252 50 Vestec, Czech Republic
E-mail: pnovak@biomed.cas.cz
Website: http://peterslab.org/
Tel.: +420 325 873 610

Significance

Even though the amine reactive BS2G and DSG cross-linkers have the same length of spacer and are based on N-hydroxysuccinimidic group, our data showed that each of them formed preferentially with different cross-links. We demonstrated that the choice of cross-linker can have a significant impact on the output data for structural characterization of biomolecules. Using equimolar mixtures of DSG with d6-BS2G, and BS2G with d6-DSG, we established that the polar BS2G preferentially bound to polar regions of modified molecule, whereas non-polar DSG bound to hydrophobic regions. This phenomenon established that the mixture of polar and non-polar cross-linkers acted as an efficient tool for the determination of distance constraints in proteins.

Keywords

Structural mass spectrometry; protein structure; chemical cross-linking; disuccinimidyl glutarate; bis(sulfosuccinimidyl) glutarate; bovine serum albumin

Author Contributions

P. Novák planned the experiments and supervised the manuscript writing. J. Fiala performed the chemical cross-linking experiments, prepared samples for MS measurements, analyzed acquired data including quantitation and wrote the manuscript. Z. Kukačka helped with the design of chemical cross-linking experiments and participated in MS data interpretation.

Acknowledgment

Declaration of interest

The authors declare that they have no conflicts of interests relating to the contents of this article.

Abstract

The combination of chemical cross-linking and mass spectrometry is currently a progressive technology for deriving structural information of proteins and protein complexes. In addition, chemical cross-linking is a powerful tool for stabilizing macromolecular complexes for single particle cryo-electron microscopy. Broad pallets of cross-linking chemistry, currently available for the majority of cross-linking experiments, still rely on the amine-reactive N-hydroxysuccinimide esters targeting mainly N-termini and lysine side chains. These cross-linkers are divided into two groups: water soluble and water insoluble; and research teams prefer one or another speculating on the benefits of their choice. However, the effect of cross-linker polarity on the outcome of cross-linking reaction has never been studied. Herein, we use both polar (bis(sulfosuccinimidyl) glutarate) and non-polar (disuccinimidyl glutarate) cross-linkers and systematically investigated the impact of cross-linker hydrophobicity on resulting distance constraints, using bovine serum albumin as a model protein.

1. Introduction

Combination of chemical cross-linking and mass spectrometry (CXMS) is a popular technique for structural characterization of proteins and their complexes. Since the inception of CXMS's use for single protein structural study two decades ago [1], it is expanding as an alternative technique to high resolution spectroscopic methods of structural biology. The advantages of CXMS are small sample requirement, no limitation in protein size, native environment working condition, high sensitivity and fast data processing. All these aspects make CXMS very effective for proteins or their complexes that are difficult to solve by crystallography or NMR alone. In the majority of experiments reported, a protein or protein complex reacted with a bifunctional cross-linker that introduced a covalent bond between residues that were in close proximity[2]. The cross-linked protein was then digested, and the generated peptide mixture was analyzed by mass spectrometry for identifying the cross-linked peptides [3]. Such distance constraints were subsequently utilized for structural model design [4–6].

CXMS is well known for generating distance constraints for building the structural models of proteins and protein complexes.[7] In this regard, a diverse repertoire of the cross-linking chemistry is currently available utilizing homobifunctional [1,8,9], heterobifunctional [9,10] and photo-active [11,12] chemical probes. The number of restraints is insufficient to build a reliable structural model *ab-initio,* if one restraint is required per ten amino acids [4,5]. To increase the number of restraints, higher amount of cross-linking probe can be considered for the reaction. However, high concentration of cross-linker triggers an artificial conformation change due to the disruption of local electrostatic interactions. Further structural perturbations are caused by stabilizing a low-populated conformation or by limiting protein flexibility. Artificial conformational change exposes previously protected site and makes it accessible to the cross-linker [13]. Such findings advocate for a novel protocol allowing the detection of sufficient number of distance constraints by keeping the concentration of cross-linker low.

A broad pallet of cross-linking chemistry, currently available for the majority of cross-linking experiment still rely on the amine-reactive N-hydroxysuccinimide (NHS) esters targeting mainly N-termini and lysine side chains. Thus, our efforts focus on these chemical probes in detail. These homobifunctional NHS-esters can be, in principle, divided into two categories: water soluble and water insoluble; and researchers preferred one or another based on their benefits. Although both hydrophilic [1,14] and hydrophobic [15,16] cross-linkers were successfully utilized to derive restraints for a design of structural model of proteins and protein complexes, the effect of cross-linker polarity on the outcome of the cross-linking reaction has not been studied yet. Herein, we used both polar (bis(sulfosuccinimidyl) glutarate, BS2G) and non-polar (disuccinimidyl glutarate, DSG) cross-linkers (Figure 1) and systematically investigated the impact of cross-linker hydrophobicity on resulting

distance constraints. Bovine serum albumin (BSA) was incubated with different mixtures consisting of regular and isotopically labeled probes, and the isotopic signature was exploited to determine the origin of identified cross-links. Subsequent quantification ratio of polar or non-polar probes in situations was done where both chemical probes form cross-links[17]. Such approach enabled the identification of five and two cross-links favoring DSG and BS2G, respectively, and 32 cross-links without significant preferences for cross-linker hydrophobicity. Based on these observations, BSA was also cross-linked using BS2G, DSG alone and with their equimolar mixture. Further, 46 and 47 cross-links were identified for BS2G and DSG, respectively, and 67 cross-links were determined for BS2G/DSG equimolar mixture. The ratio of cross-linker over the protein was kept identical to experiments where only polar or non-polar probe was taken into account.

2.   Materials and Methods

2.1. Chemicals and Materials

BSA was purchased from Merck (USA). Disuccinimidyl glutarate (DSG), bis(sulfosuccinimidyl)glutarate (BS2G), and its six deuterated analogs were bought from Creative Molecules Inc. (USA). In addition, control experiments were prepared with DSG and BS2G cross-linkers and its four deuterated analogs obtained from ProteoChem (USA). Trypsin/Lys-C mass spectrometry grade proteases from Promega Corporation (USA) were used for digestion. All solvents used for HPLC analysis were LC/MS grade, purchased from Thermo Scientific (USA). Additional chemicals reported in this paper were purchased from Merck (USA) with the highest available purity.

2.2. Chemical cross-linking

DSG, BS2G and equimolar mixtures composed of DSG/BS2G, DSG/d6-BS2G and d6-DSG/BS2G were used for modification of 10 μM solution of BSA in 20 mM HEPES, 20 mM NaCl (pH 7.5) buffer. Additionally, control experiments using mixtures of DSG/d4-DSG and BS2G/d4-BS2G were performed. All previously mentioned cross-linkers and mixtures were prepared to a concentration of 10 mg/ml using anhydrous DMSO and added to BSA solution in $100 \times$ molar excess. Reactions were done for 30 minutes at room temperature. Before digestion, disulfide bonds were reduced with 20 mM Tris(2-carboxyethyl)phosphine (TCEP) at 56 °C for 20 minutes. Free cysteines were alkylated with 20 mM iodoacetamide (IAA) at room temperature for 20 minutes in the dark. After modification, samples were diluted by 5 times with a solution of 50 mM 4-ethylmorpholine buffer (pH 8.5):AcN (80:20 v/v); subsequently, trypsin/Lys-C was added (protein:enzyme ratio 1:20). Samples were digested overnight at 37 °C. Digested mixture was quenched with TFA to a final concentration of 0.1% and subsequently dried by SpeedVac (Eppendorf, Germany). All cross-linking experiments were triplicated for consistency.

2.3. LC-MS/MS analysis

Prior to the LC-MS analysis, digestion mixture was reconstituted with 20 μl of 0.1% formic acid. For analysis, 1 μg of BSA peptide was injected and analyzed with Agilent 1200 series HPLC system equipped with desalting pre-column (Zorbax 300SB-C18 5 μm, 0.3×5 mm) followed by analytical column (Zorbax 300SB-C18 3.5 μm, 0.3×150 mm from Agilent Technologies(USA)) heated to 60°C. A flow rate of 10 μl/min with acetonitrile gradient 5%–40% with 35 minutes running time was used. The chromatographic system was directly coupled with electrospray ionization source of SolariX FT-ICR mass spectrometer (Bruker Daltonics, Germany) equipped with 15 T superconducting magnet. Eluted peptides were analyzed in positive broadband mode with 1M transient data points over the range 250–2500 m/z. Data independent mode was used while ESI-TOF tuning mix (Agilent

Technologies, USA) served as a lock mass (m/z 922.0098) and as a center of isolation window of $\pm$ 500 Da for MS/MS. Fragmentation data were acquired with 22.5 eV collision voltage, 0.2 s MS/MS ion accumulation and 2 accumulated scans per spectra.

2.4. Cross-links identification

In all the spectra, complete chromatograms were deconvoluted with SNAP 2.0 algorithm integrated in Data Analysis 4.4 (Bruker Daltonics, Germany) and exported to the mascot generic files. Obtained files were searched by StavroX 3.6.0.1 [18] with the following settings. Protease sides and modification: C-terminal cleavage of lysine, arginine and tyrosine with a maximum of 3 missed cleavages; static modification carbamidation of cysteines; variable oxidation of methionines. For all experiments, cross-linker was defined as $C_5H_4O_2$ due to the same spacer composition of DSG and BS2G. Cross-linkers specificity was set for N-terminus, lysine, serine and tyrosine. Precursor and fragmentation errors were set to 1.0 and 2.0 ppm, respectively. All cross-linked candidates were manually checked.

2.5. Relative quantification of cross-linked peptides

Ratio of light and heavy linker was determined for all identified cross-linked peptides as previously published [17]. Based on the previously established cross-links from StavroX, the most intense precursor ion (charge state) for cross-linked peptide was selected and used for the creation of extracted ion chromatogram in Data Analysis software. Final spectrum was exported as .XY file by sum of all spectra where precursor ion for light and heavy cross-linked peptide was found. The ratio of light and heavy linker was evaluated in mMass software (version 5.5.0) [19] by fitting experimental data with theoretically simulated isotopic pattern for each cross-linked peptide.

3. Results and discussion

3.1. Chemical cross-linking

The impact of cross-linker polarity on the final outcome was investigated on a well characterized BSA that was widely used as model protein for several cross-linking studies [20]. In this study, BSA was modified by equimolar mixtures of DSG ("light form") with d6-BS2G ("heavy form") and BS2G ("light form") with d6-DSG ("heavy form"). For cross-linking reactions with mixtures composed of DSG/d6-BS2G and d6-DSG/BS2G, 48 and 47 unique cross-links were identified respectively. For further evaluation, cross-links confirmed in triplicates were only considered, and each of them was confirmed in mass spectrum. Surprisingly, the observation of isotopic patterns in mass spectra of identified cross-links, revealed that the ratios of light/heavy linked peptides for some cross-links were way far from the expected ratio 50:50. To illustrate the observation of different ratios, two cross-links K211–K242 (Fig. 2A-D) and K221–K294 (Fig. 2E-H) were selected as models. Figures 2A and 2E represent spectra where cross-linking mixture of DSG/d6-BS2G was used. However, figures 2B and 2F show reciprocally labeled cross-linking mixture. The use of isotopically labeled cross-linkers enabled monitoring of cross-linking probe origin. In the case of K211–K242 cross-link, the ratio of light/heavy linked peptides revealed preferential formation of cross-linked peptides by non-polar DSG (Fig. 2A, 2B). On the contrary, cross-link K221–K294 (Fig. 2E, 2F) showed an opposite trend where modifications mainly came from polar BS2G cross-linker. Additionally, inverted ratio for DSG and BS2G was observed from two experiments where reciprocal mixtures were used.

BSA was also cross-linked with equimolar mixtures of DSG/d4-DSG or BS2G/d4-BS2G to support the previously observed phenomenon. Figures 2C, 2D and 2G, 2H show "doublet" isotopic patterns for both cross-links with expected ratio of 50:50 for light/heavy form, ensuring the correct sample preparation and chemical activities of the used cross-linking reagents. However, significant

differences were observed in absolute intensities of these spectra for non-polar DSG/d4-DSG and polar BS2G/d4-BS2G mixtures. For cross-link K211–K242, the use of non-polar mixture DSG/d4-DSG (Fig. 2C) resulted in 3.5× higher absolute intensity when compared to the polar mixture BS2G/d4-BS2G (Fig. 2D). This observation is in agreement with the previous results where DSG/d6-BS2G and reciprocal mixtures demonstrated preference for non-polar DSG over K211–K242 cross-link. The same trend was observed for K221–K294 cross-link where the use of BS2G/4d-BS2G (Fig. 2G) resulted in more than 10× higher absolute intensity when compared with non-polar DSG/d4-DSG (Fig. 2H).

3.2. Quantification of cross-links

From the 40 observed cross-links (Table S1) in both samples DSG/d6-BS2G and d6-DSG/BS2G, 39 were quantified for further investigation. The result of quantification is the ratio representing preference for the given cross-link to non-polar DSG or polar BS2G cross-linker. Figure 3A and Figure 3B represent the bar plot showing all cross-links with their calculated ratios for DSG/d6-BS2G and d6-DSG/BS2G respectively. Cross-links were sorted by the increase in polarity with less polar (left side) to the most polar (right side). The observed reciprocal pattern between Figure 3A and 3B demonstrated the credibility of sample preparation and interpretation.

According to Figure 3A and Figure 3B, half of the cross-links preferred non-polar DSG cross-linker, and only 4 cross-links preferred polar BS2G. Overall, only 25% remained close to the 50:50 ratio. Indeed, control experiments revealed 50:50 ratios of light/heavy form over all 39 quantified cross-links when mixtures of DSG/d4-DSG and BS2G/d4-BS2G were used (bar charts available in supporting material as Figure S1).

3.3. BSA structure

To explain various preferences for both cross-linking probes, it is necessary to consider the protein's chemical properties. From several reported crystal structures of BSA, the structure with the highest resolution (PDB: 4F5S) [21] was chosen for data visualization. To evaluate the polarity regions of protein, color scheme based on amino acid hydrophobicity [22] was applied to the BSA structure where red and white colors represent the hydrophobic amino acids and the hydrophilic residues, respectively. Protein was visualized in surface representation with desired transparency (Fig. 4), and all quantified cross-links were highlighted on structure connecting Cα–Cα atoms of cross-linked residues. For simplicity, only two cross-links K524–K544 and K116–K431 were chosen to demonstrate the observed phenomena. K524–K544 and K116–K431 were selected as an example for non-polar DSG preferred cross-link and polar BS2G preferred cross-link, respectively. In the case of K524–K544 (Fig. 4A), both cross-linked residues were in close proximity to many hydrophobic amino acids. Due to the restricted accessibility of polar BS2G cross-linker, K524 and K544 were preferentially connected to less polar DSG cross-linker. Unlike the previous example, cross-link K116–K431 (Fig. 4B) was situated in polar region where both cross-linked residues were surrounded by hydrophilic amino acids, thus making this part of the protein more accessible to polar BS2G cross-linker. Therefore, significant preference for BS2G cross-linker over DSG analog was expected and subsequently confirmed by our quantitative cross-linking experiment.

3.4. Venn diagrams

Based on the position in the protein structure, few cross-links were predominantly favored by non-polar or polar cross-linker as shown in Figures 3 and 4. Hence, the use of single cross-linker (DSG or BS2G) could provide inconsistent results and non-preferential cross-links could be omitted due to their

low abundance. Therefore, additional experiments were designed to establish this phenomenon. BSA was cross-linked by non-labeled DSG, BS2G and added with equimolar mixture of DSG/BS2G, keeping reaction conditions, data analysis and interpretation identical in all three cases as well as same as that of previous experimental setup. Figure 5 represents positively identified cross-links in Venn's diagrams for triplicates using DSG, BS2G and its mixture (list of common cross-links for each triplicate is available in Table S2). In principle, non-polar DSG (Fig 5A) and polar BS2G (Fig 5B) enabled the detection of 47 and 46 cross-links, respectively. However, equimolar mixture of DSG/BS2G (Fig 5C) provided 67 common cross-links (Fig. S2).

In agreement with our quantitative experiments, additional cross-links corresponded to the distance constraints that were not present in samples where only single polarity cross-linking probe was used. Therefore, usage of cross-linker mixture gained the number of identified cross-links for two reasons: some cross-links were selective for polar or non-polar cross-linking probe, and the significant increase in signal intensities facilitated successful cross-link identification in situations where the cross-links were formed by both chemical probes (polar and non-polar).

4. Conclusions

In this article, we have demonstrated that the polarity of cross-linker leaving group had a significant impact on the cross-linking results due to the difference in accessibility of cross-linking agent to reactive residues. This effect was shown on BSA protein where the origin of cross-linker was tracked by the use of isotopically labeled mixtures of DSG/d6-BS2G and d6-DSG/BS2G. Relative quantification of cross-linked peptides revealed cross-links, which were predominantly modified by non-polar DSG or polar BS2G. Additionally, preferences were correlated to the BSA structure, where polar regions were predominantly modified by polar BS2G cross-linker and vice versa. To support our findings, BSA was cross-linked with non-labeled DSG, BS2G and DSG/BS2G equimolar mixture. The DSG/BS2G mixture provided 20 and 21 extra cross-links compared to the experiments where only non-polar DSG and polar BS2G were used, respectively.

The minimal number of distance constraints required for ab initio protein structural model design was one restraint per ten amino acids [4,5]. In many cases, it was hard to reach the desired number using cross-linker targeting primary amino groups, even with the higher amounts of lysine residues. Indeed, the number of available residues, and mainly the lysine accessibility and reactivity, defined the success of cross-linking reaction [23]. Considering these limitations, it was suggested to block the most reactive lysine residues by NHS-acetate prior to the cross-linking reaction or increase the extent of cross-linker over the protein [24]. This idea became obsolete since excessive cross-linking or introduction of negative charge on lysine induced structural changes and potentially led to the creation of artificial cross-links [13]. To avoid this effect, the mixture of non-polar/polar cross-linker in slight excess could be used within structural perturbation.

Taken together, these findings highlighted the role of cross-linker polarity for designing protein cross-linking experiments and provided new insights into sample preparation where cross-linker polarity played an important role.

5. References

(1) Young, M. M.; Tang, N.; Hempel, J. C.; Oshiro, C. M.; Taylor, E. W.; Kuntz, I. D.; Gibson, B. W.; Dollinger, G. High Throughput Protein Fold Identification by Using Experimental Constraints Derived from Intramolecular Cross-Links and Mass Spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (11), 5802–5806.

(2) Farmer, T. B.; Caprioli, R. M. Assessing the Multimeric States of Proteins: Studies Using

Laser Desorption Mass Spectrometry. *Biol. Mass Spectrom.* **1991**, *20* (12), 796–800.

(3)   Rossi, V.; Gaboriaud, C.; Lacroix, M.; Ulrich, J.; Fontecilla-Camps, J. C.; Gagnon, J.; Arlaud, G. J. Structure of the Catalytic Region of Human Complement Protease C1s: Study by Chemical Cross-Linking and Three-Dimensional Homology Modeling. *Biochemistry* **1995**, *34* (22), 7311–7321.

(4)   Havel, T. F.; Crippen, G. M.; Kuntz, I. D.; Blaney, J. M. The Combinatorial Distance Geometry Method for the Calculation of Molecular Conformation II. Sample Problems and Computational Statistics. *J. Theor. Biol.* **1983**, *104* (3), 383–400.

(5)   Havel, T. F.; Kuntz, I. D.; Crippen, G. M. The Combinatorial Distance Geometry Method for the Calculation of Molecular Conformation. I. A New Approach to an Old Problem. *J. Theor. Biol.* **1983**, *104* (3), 359–381.

(6)   Rappsilber, J. The Beginning of a Beautiful Friendship: Cross-Linking/Mass Spectrometry and Modelling of Proteins and Multi-Protein Complexes. *J. Struct. Biol.* **2011**, *173* (3), 530–540.

(7)   Sinz, A. The Advancement of Chemical Cross-Linking and Mass Spectrometry for Structural Proteomics: From Single Proteins to Protein Interaction Networks. *Expert Review of Proteomics*. December 16, 2014, pp 733–743.

(8)   Kruppa, G. H.; Schoeniger, J.; Young, M. M. A Top down Approach to Protein Structural Studies Using Chemical Cross-Linking and Fourier Transform Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17* (2), 155–162.

(9)   Novak, P.; Kruppa, G. H. Intra-Molecular Cross-Linking of Acidic Residues for Protein Structure Studies. *Eur. J. Mass Spectrom. (Chichester, Eng).* **2008**, *14* (6), 355–365.

(10)   Jacobsen, R. B.; Sale, K. L.; Ayson, M. J.; Novak, P.; Hong, J.; Lane, P.; Wood, N. L.; Kruppa, G. H.; Young, M. M.; Schoeniger, J. S. Structure and Dynamics of Dark-State Bovine Rhodopsin Revealed by Chemical Cross-Linking and High-Resolution Mass Spectrometry. *Protein Sci.* **2006**, *15* (6), 1303–1317.

(11)   Suchanek, M.; Radzikowska, A.; Thiele, C. Photo-Leucine and Photo-Methionine Allow Identification of Protein-Protein Interactions in Living Cells. *Nat. Methods* **2005**, *2* (4), 261–267.

(12)   Gomes, A. F.; Gozzo, F. C. Chemical Cross-Linking with a Diazirine Photoactivatable Cross-Linker Investigated by MALDI- and ESI-MS/MS. *J. Mass Spectrom.* **2010**, *45* (8), 892–899.

(13)   Rozbeský, D.; Rosůlek, M.; Kukačka, Z.; Chmelík, J.; Man, P.; Novák, P. Impact of Chemical Cross-Linking on Protein Structure and Function. *Anal. Chem.* **2018**, *90* (2), 1104–1113.

(14)   Schulz, D. M.; Ihling, C.; Clore, G. M.; Sinz, A. Mapping the Topology and Determination of a Low-Resolution Three-Dimensional Structure of the Calmodulin-Melittin Complex by Chemical Cross-Linking and High-Resolution FTICRMS: Direct Demonstration of Multiple Binding Modes. *Biochemistry* **2004**, *43* (16), 4703–4715.

(15)   Rozbesky, D.; Sovova, Z.; Marcoux, J.; Man, P.; Ettrich, R.; Robinson, C. V; Novak, P. Structural Model of Lymphocyte Receptor NKR-P1C Revealed by Mass Spectrometry and Molecular Modeling. *Anal. Chem.* **2013**, *85* (3), 1597–1604.

(16)   Rappsilber, J.; Siniossoglou, S.; Hurt, E. C.; Mann, M. A Generic Strategy To Analyze the Spatial Organization of Multi-Protein Complexes by Cross-Linking and Mass Spectrometry. *Anal. Chem.* **2000**, *72* (2), 267–275.

(17)   Kukacka, Z.; Rosulek, M.; Strohalm, M.; Kavan, D.; Novak, P. Mapping Protein Structural

Changes by Quantitative Cross-Linking. *Methods* **2015**, *89*, 112–120.

(18)   Götze, M.; Pettelkau, J.; Schaks, S.; Bosse, K.; Ihling, C. H.; Krauth, F.; Fritzsche, R.; Kühn, U.; Sinz, A. StavroX-A Software for Analyzing Crosslinked Products in Protein Interaction Studies. *J. Am. Soc. Mass Spectrom.* **2012**, *23* (1), 76–87.

(19)   Strohalm, M.; Kavan, D.; Novák, P.; Volný, M.; Havlíček, V. MMass 3: A Cross-Platform Software Environment for Precise Analysis of Mass Spectrometric Data. *Anal. Chem.* **2010**, *82* (11), 4648–4651.

(20)   Iacobucci, C.; Piotrowski, C.; Aebersold, R.; Amaral, B. C.; Andrews, P.; Bernfur, K.; Borchers, C.; Brodie, N. I.; Bruce, J. E.; Cao, Y.; et al. First Community-Wide, Comparative Cross-Linking Mass Spectrometry Study. *Anal. Chem.* **2019**, *91* (11), 6953–6961.

(21)   Bujacz, A. Structures of Bovine, Equine and Leporine Serum Albumin. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2012**, *68* (10), 1278–1289.

(22)   Eisenberg, D.; Schwarz, E.; Komaromy, M.; Wall, R. Analysis of Membrane and Surface Protein Sequences with the Hydrophobic Moment Plot. *J. Mol. Biol.* **1984**, *179* (1), 125–142.

(23)   Novak, P.; Kruppa, G. H.; Young, M. M.; Schoeniger, J. A Top-down Method for the Determination of Residue-Specific Solvent Accessibility in Proteins. *J. Mass Spectrom.* **2004**, *39* (3), 322–328.

(24)   Guo, X.; Bandyopadhyay, P.; Schilling, B.; Young, M. M.; Fujii, N.; Aynechi, T.; Guy, R. K.; Kuntz, I. D.; Gibson, B. W. Partial Acetylation of Lysine Residues Improves Intraprotein Cross-Linking. *Anal. Chem.* **2008**, *80* (4), 951–960.