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Selective, Bead-Based Global Peptide Capture Using a Bifunctional Cross-Linker

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Abstract

Peptides are important species for a variety of biological functions. Detection and analysis of these molecules can be complicated by the presence of background matrix or contaminants. Therefore, a selective method to capture peptides could provide researchers with an option to isolate these remarkable species. Our goal was to perform a set of experiments that would validate the concept of a novel, selective peptide capture, whereby peptides are isolated on functionalized magnetic beads through the use of the heterobifunctional cross-linker, Sulfo-LC-SPDP. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to monitor mass changes associated with the cross-linking reaction. MALDI-TOF MS was then used to monitor conjugation between the cross-linked peptides and sulfhydryl magnetic beads by analyzing supernatant solutions for the presence or absence of cross-linked peptide. Through these experiments, we have proof of concept data confirming that peptides can be isolated on sulfhydryl magnetic beads by using Sulfo-LC-SPDP. This method is a suitable selective global peptide isolation strategy to separate the molecules from contaminating species or sample matrix. This novel method has a variety of potential applications and detection methods.

One of the central goals of analytical chemistry is to separate species of interest from their surrounding matrices. In order to selectively capture a species, one must take advantage of

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Associated Content: Supporting Information: Supporting Information 1: MALDI-TOF analysis of the negative control peptide with the cross-linking reaction and sulfhydryl magnetic beads. This material is available free of charge via the Internet at <http://pubs.acs.org>.

distinct chemical differences that exist between that species and the background matrix or other contaminants. Peptides are an excellent example of analytes of interest that are commonly found in complicated biological matrices. Peptides are chemical species with diverse and critical biological functions. For example, these molecules have been shown to have integral roles in hormone regulation and neurological signaling.¹

In this document, we describe a novel approach for a selective global capture of peptide species using a heterobifunctional cross-linker and functionalized magnetic beads. The cross-linker, Sulfo-LC-SPDP (Thermo, Rockford, IL), has previously been used to manipulate and immobilize proteins on a variety of different surfaces.^{2–6} Functionalized magnetic beads enable easy and rapid separations.^{7,8} For the application evaluated in this study, Sulfo-LC-SPDP is paired with sulfhydryl magnetic beads resulting in a novel strategy for selectively capturing peptides from a variety of different matrices.

In order to capture the peptides, we are exploiting the presence of amine groups on the N-terminus of the peptide and lysine residue side chains. The heterobifunctional cross-linker, Sulfo-LC-SPDP (Figure 1A), can be utilized for such a purpose. When used for our application, free amines found on peptides displace the *N*-hydroxysuccinamide (NHS) ester leaving group on one end of the cross-linker, forming an amide bond (Figure 1B). The opposite end of the cross-linker features a reactive 2-pyridyldithio group that can be easily cleaved leaving a chemically advantageous sulfhydryl.^{9,10} A new disulfide bond can be introduced between the cross-linked peptide and sulfhydryl coated magnetic beads (Figure 1C). This reaction immobilizes the cross-linked peptides on the beads. The conjugated cross-linked peptide and bead can then be subjected to washings and other manipulation steps with the use of capture magnets outside of the sample vial, thus enabling comprehensive enrichment. After successful isolation, the disulfide bond between the cross-linked peptide and the magnetic bead is cleaved using dithiothreitol (DTT) resulting in a population of free, cross-linked peptides. Our goal was to validate this novel method to isolate peptides using the aforementioned chemistry while monitoring the reactions with matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Figure 1D). This method is appropriate for global capture of all amine containing species of interest. The chemistry is intended to be used for the isolation of peptides or proteins from a contaminant such as a detergent or polymer.

Materials and Methods

Reagents and Materials

Synthetic peptides were produced by GenScript (Piscataway, NJ). The heterobifunctional cross-linker, Sulfo-LC-SPDP, was purchased from Thermo (Rockford, IL). Dialysis tubing was obtained from Spectrum Laboratories (Rancho Dominguez, CA). BcMag Sulfhydryl-Terminated-Magnetic Beads were acquired from Bioclone, Inc. (San Diego, CA). Mass spectrometry grade solvents including acetonitrile (ACN) and water were obtained from Burdick and Jackson (Muskegon, MI). Peptide Calibration Standard was purchased from Bruker (Billerica, MA). All unspecified reagents were acquired from Sigma Aldrich (St. Louis, MO).

Sample Preparations

Peptides (1 $\mu\text{g}/\mu\text{L}$) in water with an equivalent volume of phosphate buffered saline (PBS; pH 7.2) were reacted with Sulfo-LC-SPDP (10 $\mu\text{g}/\mu\text{L}$) for at least 45 min at ambient temperature following the manufacturer's instructions. Molar ratios for the cross-linking reactions were determined on the basis of the molecular weight of the reacted peptide. The reaction mixture (approximately 25 μL) was dialyzed against 1000 $\times$ volume of PBS for 3 h using 500–1000 Da cutoff cellulose ester dialysis tubing to remove excess Sulfo-LC-SPDP. After the initial 3 h, buffer was exchanged for 1000 $\times$ volume of nanopure water and dialysis continued overnight. Samples were removed from the tubing and dried using a vacuum concentrator. Dried samples were resuspended in PBS prior to magnetic bead isolation.

Sulfhydryl coated magnetic beads were prepared according to the manufacturer's instructions (Bioclone). The beads were suspended in PBS (30 mg/mL), and 200 μL of the bead solution was washed with three volumes of 200 μL of PBS. A magnetic separator was used to immobilize the beads in order to remove the supernatant. Beads were then incubated in 200 μL of DTT (100 mM) for 15 min at ambient temperature; supernatant was removed, and beads were again washed 3 times with PBS. Cross-linked peptide samples were incubated overnight with the prepared beads at 4 °C. Supernatants from the incubation and three subsequent PBS washes were collected to determine conjugation efficiency. Peptides were released from the beads by incubation with 200 μL of DTT (100 mM). Supernatants from the release incubation and three subsequent PBS washes were collected for MALDI-TOF analysis.

Proteins (20 μg), extracted from the SW480 colon cancer cell line, in the presence of optimal cutting temperature (OCT) compound were subjected to an in-solution tryptic digestion. Briefly, the sample was reduced with 5 mM DTT for 60 min at 56 °C, alkylated with 10 mM IAA at ambient temperature in the dark for 20 min, and incubated with trypsin (1 $\mu\text{g}/\mu\text{L}$) at a 1:20 trypsin/protein ratio overnight at 37 °C. The resulting peptide sample was spiked with 0.47 nmol of bradykinin to determine conjugation efficiency. The sample volume was reduced to 20 μL and reacted with an equivalent volume of PBS and 20 μL of Sulfo-LC-SPDP (10 $\mu\text{g}/\mu\text{L}$) for 60 min at ambient temperature. The reaction mixture was desalted with C18 ZipTips, dried, and resuspended in PBS. Complex cross-linked peptide sample was incubated overnight at 4 °C with 150 μL of prepared beads. Supernatants and washes were collected as previously described. Peptides were released from the beads by incubation with 150 μL of DTT (100 mM). Supernatants from PBS washes were collected.

Samples were desalted with C18 ZipTips (Millipore, Billerica, MA) and dried to approximately 1 μL before being hand spotted on the MALDI target. An equal volume of 2,5-dihydroxy benzoic acid (DHB) (15 mg/mL in 50/50 ACN/0.1% formic acid) was used for matrix. A Bruker AutoFlex III MALDI-TOF (Bremen, Germany) operated in positive-ion mode with a source voltage of 19.00 kV was used to collect mass spectrometric data. Reflector mode (21.00 kV) was used to analyze all samples setting a mass range of 400–5000 Da. Laser (337 nm N_2) focus was set to “default”, and 1000 shots were fired at each sample spot (100 Hz). Ion suppression was set to 500. FlexAnalysis, version 3.0 (Bruker), was used to analyze MS data and generate spectra.

Results and Discussion

The first step of this selective peptide capture is conjugation of the peptide to the heterobifunctional cross-linker, Sulfo-LC-SPDP (Figure 1A). All experiments were first conducted with a standard peptide, bradykinin (RPPGFSPFR), for proof of concept. Bradykinin was chosen for this method development because it is commonly found in standard peptide mixes and is readily available. Parameters adjusted to increase conjugation efficiency included reaction incubation time, mixing during incubation, and molar ratio of peptide to Sulfo-LC-SPDP. Molar ratio was the only parameter to have a significant impact on reaction efficiency. Cross-linker product information suggests that one mole of peptide requires between 15 and 25 mols of Sulfo-LC-SPDP for complete conjugation. To minimize reagent costs, smaller reaction ratios (1:1, 1:2, 1:5, and 1:10) were analyzed. Molar ratios of both 1:2 and 1:5 bradykinin to Sulfo-LC-SPDP have shown 100% conjugation efficiency; however, reactions at a 1:2 ratio are not as reproducible as the 1:5 ratio. Given the reproducibility of 100% cross-linker reaction efficiency and minimal waste, the 1:5 molar ratio was deemed optimal (Figure 2).

Conjugation efficiency was monitored using MALDI-TOF MS to observe an appropriate mass shift, consistent with peptide-cross-linker reaction. Without any modification, bradykinin has a mass of 1060 Da (Figure 2A). Cross-linker conjugation adds 311.4 Da to the peptide shifting the m/z signal to 1371. However, while monitoring the reaction, repeated signals corresponding to the addition of 202.3 Da to bradykinin were observed ($m/z = 1261$). This mass difference corresponds to conjugation with Sulfo-LC-SPDP with loss of the pyridine-2-thione portion of the cross-linker. Loss of this portion of the molecule does not have any negative implications for subsequent steps in this selective enrichment. However, it means that, for any given peptide with a single primary amine, both the +311.4 and +202.3 peaks can be detected, which marginally complicates the data analysis (Figure 2B).

To show that this selective peptide enrichment method reacts specifically, a synthetic peptide with a primary sequence of FDTLYGPVSAEGTM was purchased to serve as a negative control (GenScript, Piscataway, NJ) (Figure 2C). The peptide features an acetylated N-terminus, no free amines, and no sulfhydryl groups and thus should not react with the heterobifunctional cross-linker. Reaction of the negative control peptide ($m/z = 1551$) in a 1:10 molar ratio with Sulfo-LC-SPDP results in no mass shift for the peptide, indicating the cross-linker conjugation is specific for amine groups (Figure 2D).

Conjugating cross-linked peptides to sulfhydryl coated magnetic beads is the second step in this isolation method. To determine the efficiency of disulfide bond formation between the cross-linked peptide and magnetic beads, cross-linked bradykinin samples were incubated with the beads and washed with PBS. Supernatant solutions were collected using a capture magnet to isolate beads from solution. The supernatants were analyzed via MALDI-TOF MS for the presence of peptide and/or cross-linked peptide. When a disulfide bond has been formed, cross-linked peptide should not be detected in the supernatant. Upon reduction of the disulfide bond using DTT, cross-linked peptide is released from the magnetic bead and can be detected. Subsequent postcleavage washes were analyzed and collected to determine the method that would yield maximum recovery. The incubation solution showed some

cross-linked bradykinin signal (Figure 3A), indicating that capture of the cross-linked peptides by the beads is not complete. However, washes of the beads showed no evidence of cross-linked bradykinin (Figure 3B). Addition of DTT to the bead solution cleaved the disulfide bond between the magnetic beads and cross-linked bradykinin, resulting in cross-linked bradykinin detected via MALDI-TOF MS (Figure 3C). The presence of cross-linked bradykinin signal prior to the disulfide bond cleavage indicates that not all of the cross-linked peptide has been conjugated to the beads and could be further optimized to improve conjugation efficiency. In this proof-of-concept experiment, we have demonstrated that heterobifunctional amine and sulfide reactive cross-linkers, coupled with sulfide reactive magnetic beads, provide a feasible route to selectively capturing peptides.

To validate the selectivity of the sulfide reactive magnetic beads, the negative control peptide was also reacted with the sulfhydryl magnetic beads using the previously described reaction and analysis methods for bradykinin. Supernatant from the bead incubation and PBS washes show decreasing signals for this peptide with each subsequent wash. These results indicate that the peptide does not form a disulfide bond with the sulfhydryl coated magnetic beads, and the method is specific for cross-linked peptides (Supporting Information 1).

In mass spectrometry-based proteomic experiments, trypsin is commonly used to generate peptides from proteins. Due to the specificity of trypsin, proteolytic cleavage occurs after arginine and the lysine residues. Many of these peptides would have two available free amines, peptide N-terminus and lysine side chain, to react with Sulfo-LC-SPDP. In order to determine the compatibility of this peptide capture method with tryptic peptides or similar peptides with multiple free amines, a synthetic “tryptic mimic” (FTATDSHTDTPEALTTK) featuring a C-terminal lysine was obtained (GenScript). The unreacted peptide has an m/z signal at 1836 (Figure 4A). Reaction ratio with Sulfo-LC-SPDP was optimized to account for the additional amine group. Conjugation reactions were monitored for appropriate m/z shift corresponding to the addition of two cross-linking molecules (+622.8, +513.7, and +404.6). Reproducible, efficient reactions for both amine sites were observed after using a molar ratio of 1:25 peptide to cross-linker (Figure 4B). The most common addition was +404.6, which generated a signal at approximately 2238 m/z . In a similar manner as bradykinin, the “tryptic mimic” peptide was able to bind to and be released from the sulfhydryl magnetic beads utilizing Sulfo-LC-SPDP (Figure 4). This interaction is proven through the lack of signal in a PBS wash followed by return of signal at 2238 m/z after incubation with DTT to reduce the disulfide bond freeing the peptide from isolation on the bead.

To assess the applicability of this method for removing contaminants from complex biological samples, a mammalian protein lysate sample spiked with the known MS contaminant, optimal cutting temperature compound (OCT),¹¹ was subjected to this novel peptide capture method. The contaminant, OCT, can be viewed in Figure 5A, as series of repeated signals, spanning from 1100 to 1900 m/z , indicating the presence of polyethylene glycol (PEG) polymer. The sample was also spiked with bradykinin (1060.3 m/z) as a positive control to monitor the efficiency of the Sulfo-LC-SPDP cross-linking reaction (Figure 5A). Successful and efficient cross-linker reaction is seen in Figure 5B through the

presence of the cross-linked bradykinin signal (1261.9 m/z) and lack of uncross-linked bradykinin signal (1060.3 m/z). Prior to peptide release (Figure 5C), cross-linked bradykinin is detected indicating that not all of the cross-linked peptides are conjugated to the sulfhydryl magnetic beads. These results are consistent with the previously described experiments. Purified captured peptides are detected without the presence of OCT after release from the sulfhydryl beads (Figure 5E). These results showcase the ability of this method to capture and isolate a biologically significant sample from a common contaminant.

Conclusions

Through this study, we have demonstrated that peptides can be selectively conjugated with the heterobifunctional cross-linker, Sulfo-LC-SPDP, immobilized on sulfhydryl coated magnetic beads, and analyzed with mass spectrometry. Our results validate the concept of this novel method for use with peptides featuring both single and multiple amines, including tryptic peptides. Although conditions might need to be optimized for specific experimental requirements, we anticipate that this method could be utilized for a variety of applications where peptides need to be isolated from a background matrix or sample contaminant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Reactions utilized for this selective peptide capture method. The heterobifunctional cross-linker, Sulfo-LC-SPDP, is shown in blue. (A) Primary amines on the peptide react with the NHS ester of Sulfo-LC-SPDP. (B) The reactive 2-pyridyldithio group on the opposite end of Sulfo-LC-SPDP can be displaced resulting in a sulfhydryl. (C) Cross-linked peptide with a free sulfhydryl can be immobilized on sulfhydryl coated magnetic beads. (D) After sufficient enrichment, the disulfide bond between the cross-linked peptide and the magnetic bead can be cleaved. This leaves modified peptides that can be detected via MS.

Figure 2.

Peptide conjugation with Sulfo-LC-SPDP. Bradykinin (A, B) and negative control synthetic peptide (C, D) before (A, C) and after (B, D) conjugation with the heterobifunctional cross-linker, Sulfo-LC-SPDP in a 1:10 peptide to cross-linker molar ratio. Conjugation with the cross-linker and loss of the pyridine-2-thione group results in a mass increase of 202.3 or 311.4 Da. The lack of m/z change with the negative control peptide (C, D) highlights the specificity of this reaction.

Figure 3. MALDI-TOF MS analysis of sulfhydryl magnetic bead isolation. (A) Cross-linked bradykinin was incubated with sulfhydryl magnetic beads overnight at 4 °C. (B) After removal of the supernatant, beads were washed with PBS. (C) Beads were incubated with DTT to release the cross-linked peptide, and washed again with PBS (D). Sample solutions were analyzed for the presence of cross-linked bradykinin. The absence of signal in the PBS wash supernatant, followed by signal in the DTT incubation supernatant and subsequent wash indicates that cross-linked bradykinin is immobilized on the sulfhydryl magnetic beads.

Figure 4.

MALDI-TOF analysis of the tryptic mimic peptide with the cross-linking reaction and sulfhydryl magnetic beads. The tryptic mimic peptide (A) was reacted with Sulfo-LC-SPDP. (B) Successful, efficient conjugation with two cross-linking molecules occurred resulting in an m/z shift. (C) Cross-linked peptide was then incubated with sulfhydryl magnetic beads. (D, E) After keeping the supernatant from the incubation, beads were washed three times with PBS. (F, G) Cross-linked peptide was released from the magnetic beads through incubation with DTT and collected through subsequent PBS washes. This analysis highlights the ability of this selective bead-based method to isolate peptides with more than one primary amine.

Figure 5.

Biological application of peptide capture method. A complex biological sample with known contaminant, OCT, (A) was reacted with Sulfo-LC-SPDP. (B) Efficient cross-linking is evidenced by the m/z shift for bradykinin. (C) Cross-linked sample was then incubated with sulfhydryl magnetic beads. (D) Subsequent washes with PBS removed the contaminant. (E) Release of cross-linked peptides with DTT. This analysis highlights the ability of this method to capture and release peptides of a complex biological sample containing a contaminant.