Bioconjugate Chemistry

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Development of a Potential High-Throughput Workflow to Characterize Sites of Bioconjugation by Immuno-Affinity Capture Coupled to MALDI-TOF Mass Spectrometry

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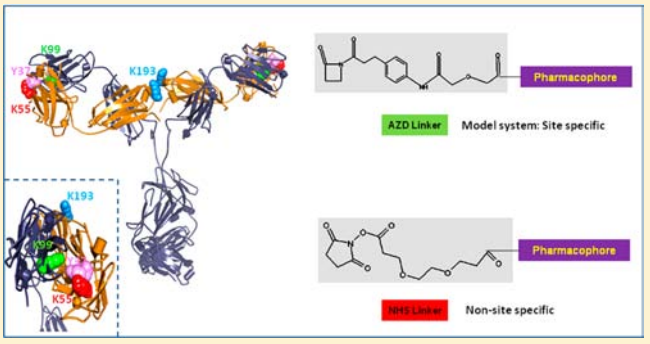
Supporting Information

ABSTRACT: The characterization of conjugation sites in bioconjugates is critical in the early discovery phase because site-specific conjugation improves in vivo stability and drug efficacy. We previously developed an engineered monoclonal antibody (mAb) scaffold which enables site-specific conjugation toward a reactive lysine (Lys) residue on each heavy chain (HC) by using an azetidinone (AZD) linker. In order to explore conjugations in other location which avoids potential interference with target binding, other chemical linkers have been studied and the investigation of *N*-hydroxysuccinimade (NHS) linker is reported here. The complexity of identifying the sites lies in part to the large number of Lys residues available for conjugation on the mAb scaffold. This has posed technical challenges to standard peptide mapping approaches. Therefore, an alternative strategy intended for a rapid analysis has been investigated by coupling immuno-affinity capture to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). In this study, we have employed a novel application of two different capture formats: Surface enhanced laser dissociation/ionization (SELDI) and mass spectrometry immunoassay (MSIA) tips to reduce the analysis time. An antibody against the pharmacophore portion was immobilized to capture the conjugated peptides, and subsequently provide characterization of the conjugation sites on the scaffold. Multiple sites for the AZD and NHS linkers have been easily identified and confirmed by MS2 sequencing. Lysine99 is the predominant site for the AZD linker, and Lysine55 is the primary site for the NHS linker with Lysine193 and Tyrosine37 being minor sites as shown in the abstract figure. We have also demonstrated the use of conjugation mapping to compare the distribution pattern between the AZD and NHS linkers as well as to study the stability of conjugation sites in a rapid way.



INTRODUCTION

Bioconjugates which attach therapeutic peptides, toxins, or proteins via chemical linkers to a monoclonal antibody (mAb) scaffold represent a class of promising biotherapeutics to fight against cancer and other diseases. The improved drug efficacy lies in the combined advantages from the mAb scaffold and the effectors. It is critical to identify sites of conjugation so as to facilitate protein engineering to obtain final bioconjugates with improved site specificity, linker stability, and drug efficacy. The advantage of achieving site-specific conjugation has been demonstrated by in vivo evidence[1,2] in terms of improved therapeutic index.

The linker plays a key role in bioconjugates and are generally designed to have high affinities toward either Lysine (Lys) or Cysteine (Cys) residues.[3] We have developed a technology to enable site-specific conjugation to a particular reactive Lys on each heavy chain (HC) of an engineered mAb termed CVX-2000[4,5] by using an azetidinone (AZD) linker. On the basis of this platform, we have been devoting efforts to explore other chemical linkers that are able to attach more effectors such as a combination of therapeutic peptides, toxins, and proteins. In this regard, the conjugation pattern of *N*-hydroxysuccinimide (NHS) linker has been investigated in this study. The NHS linker reacts primarily with Lys residues to form an amide bond; however, it has been reported to target multiple residues on other proteins.[6] The degree of conjugation site specificity of this linker toward general mAb scaffolds is largely unknown based on current literature and is estimated to result in heterogeneous conjugation species.

Received: July 31, 2012
Revised: November 21, 2012
Published: November 27, 2012

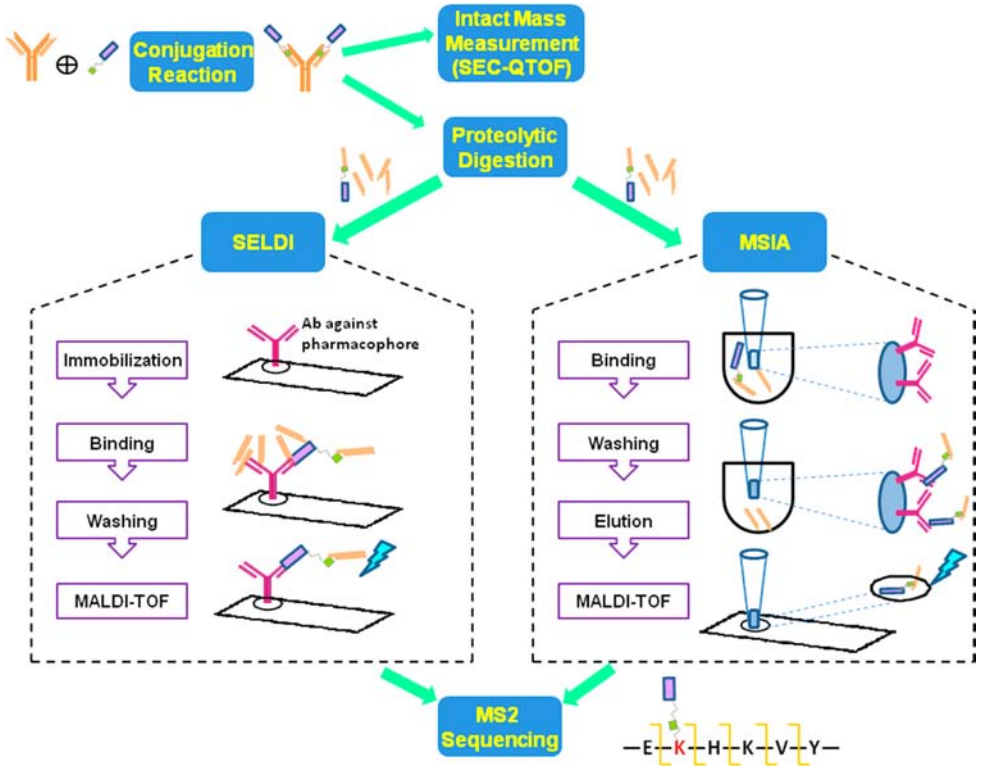


Figure 1. Experimental workflow.

The identification of Lys conjugation sites has posed a great challenge because of the frequent occurrence of this amino acid residue in a typical Ab. Liquid chromatography–mass spectrometry (LC-MS) based peptide mass fingerprinting (PMF) is currently the standard analytical method for this purpose by comparing the chromatograms between the unconjugated and the conjugated peptides after enzymatic digestion.[7] However, this peptide mapping process is time-consuming and laborious due to long gradient time needed for separation and the extensive search for a characteristic mass shift, although database searching facilitated by in silico digestion of the known Ab sequence can shorten the analyzing time to some degree. Other LC-MS based strategies have been investigated elsewhere, such as the use of neutral loss to identify fluorescent dye attachment sites[8] and the use of precursor scan to assign cross-linked sites.[9] These methods further reduce the processing time; however, long chromatographic time is still unavoidable for maximum sequence coverage. Extra effort is also needed in the method development stage to optimize collision energy. Also, these strategies may not be directly applicable to determine conjugation sites in our case, as there are 44 discrete lys residues present in CVX-2000.

Thus, alternative strategies which can locate conjugation sites more quickly have been investigated in our study. Immuno-affinity capture serves as a great tool to selectively isolate the targets based on specific binding between Ab and antigen (Ag). The coupling of immuno-affinity capture to LC-MS has been applied to evaluate bioconjugates in vivo stability and has been demonstrated as a sensitive assay to identify metabolites from pharmacokinetic samples.[10,11] In addition to the coupling to LC-MS, immuno-affinity capture has also been combined with MALDI-TOF MS in which surface-enhanced laser desorption/ionization (SELDI) is a widely adopted technology. In SELDI, which originated in early 1990s, the surface of the sample plate for MS detection has a separation/purification function that can be manipulated with a variety of chromatographic or affinity probes based on different types of analyte–surface interactions.[12,13] The major application of SELDI lies in the proteomics and biomarker discovery field which utilizes immobilized Ab to extract potential targets from clinical samples and enables a direct on-spot MS analysis.[14−17] An alternative coupling format has been developed by immobilizing Ab onto pipet tips, termed mass spectrometric immunoassay (MSIA).[18,19] They function as microcolumns[20] to purify targets that can be eluted directly onto standard MALDI plate under acidic conditions. Promising qualitative results have been reported; relative and absolute quantification capabilities have also been demonstrated for the detection of biomarkers in clinical samples.[21−24] The coupling of immuno-affinity capture with MALDI-TOF MS (either the SELDI or MSIA assay) has become our preferred solution because it offers a unique speed advantage over LC-MS in terms of greatly reduced instrument time. In this study, the Ab against the pharmacophore portion, which is the same across the AZD and NHS bioconjugates, was immobilized to capture the conjugated peptides after digestion. The Ab scaffold peptide can be then inferred based on the mass detected in the SELDI and the MSIA spectra together with our prior knowledge of CVX-2000 sequence. This represents a novel application of these two assays to quickly identify conjugation sites.

High-resolution MS is commonly used to achieve high confidence regarding peptide identity, but does not provide information to determine which residue is modified given that the NHS linker may not be limited to Lys residues. Therefore, MS2 was used in our study to locate the actual sites in addition to resolving sequence ambiguity resulting from two peptides with a mass difference less than 0.01 Da. The conjugated peptides are essentially branched peptides, and current

dx.doi.org/10.1021/bc300413c | *Bioconjugate Chem.* 2013, 24, 53−62

sequencing techniques to identify the branched/cross-linked sites include high-resolution LC-MS/MS by LTQ-FTMS,[25] post-source decay by MALDI-TOF,[26] and TOF/TOF,[6] which was used in this study. Interpretation of the MS2 spectra generated by such branched peptides is always more complicated than of linear peptides due to the exponentially increased number of possible product ions. A simplified peak interpreting strategy has been employed by comparing the experimental spectra and the theoretical spectra of the potential conjugated peptide inferred from the SELDI and the MSIA assays.

Figure 1 provides a schematic view illustrating the overall experimental workflow from performing conjugation reactions and immuno-affinity capture assays to the identification and confirmation of conjugation sites. This workflow was utilized to compare the SELDI and MSIA assay capability to identify conjugated peptides for the AZD and NHS linkers under different conjugation conditions. Once the sites have been determined, a direct comparison of the total digests by MALDI-TOF MS was employed to evaluate the relative conjugation profiles between the AZD and NHS linkers. The dynamic range of the MSIA assay has also been measured based on relative quantification. In addition, a stability indicating application has been exemplified to assess labile conjugation sites under a stressed condition. Overall, our study has demonstrated a novel application of the SELDI and MSIA assays to quickly map Ab conjugation sites.

EXPERIMENTAL PROCEDURES

Synthesis and Purification of Pharmacophore-Linker. The therapeutic peptide termed pharmacophore was attached to the AZD and NHS linkers on their N-terminus as shown in the abstract figure. The end product (referred to as pharmacophore-linker) was then purified by reversed-phase liquid chromatography (RPLC). High-resolution MALDI-TOF spectrum has been obtained for each phamacophore-linker to confirm the purity and the identity (Figure 1S). The molecular formulas are $C_{60}H_{97}N_{15}O_{15}$ and $C_{56}H_{96}N_{14}O_{17}$ for the AZD and NHS pharmacophore-linkers and the theoretically calculated monoisotopic masses ($[M+H]^+$) are 1268.736 and 1237.715, respectively. The mass accuracy of the detected peak (Figure 1S) is within 30 ppm in each case. The two batches of purified pharmacophore-linker were lyophilized and stored in a desiccant bottle at −20 °C for future use.

Conjugation Reactions. Conjugation reactions were performed at 4:1 and 10:1 pharmacophore-linker to Ab scaffold molar ratio, and the reaction mixture was incubated overnight for both linkers. The 18.0 mg/mL CVX-2000 stock solution was stored in a buffer containing 10 mM histidine, 200 mM glycine, 2% sucrose, pH 6.5. For AZD conjugation, the pharmacophore-linker was dissolved in water at 10.0 mg/mL and then combined with CVX-2000. For NHS conjugation, the CVX-2000 stock was first buffer-exchanged to another formulation buffer containing 20 mM sodium acetate, 200 mM trehalose, pH 5.5 using a 30k MWCO Amicon Ultra Centrifugal Filters (Millipore) at room temperature. Then, a 10× buffer of 0.6 M sodium phosphate, pH 7.7, was spiked to increase the pH of the reaction mixture to 7.5. The pharmacophore-linker was dissolved in water at 10.0 mg/mL, and immediately added to the CVX-2000 solution.

Intact Mass Measurement by SEC-qTOF MS. Bioconjugates were first deglycosylated by glycerol-free PNGaseF (New England Biolabs) at 1:50 (w/w) enzyme to substrate ratio and incubated at 37 °C for 4 h. Subsequently, the deglycosylated samples were diluted to 2 mg/mL by HPLC-grade water and injected onto a polymeric PLRP-S column (250 × 1 mm, 5 μm, 1000 Å, Higgins Analytical) operated in size exclusion chromatography (SEC) mode with Waters Acquity UPLC system at a flow rate of 50 μL/min: 50% mobile phase A (water with 0.1% formic acid) and 50% mobile phase B (acetonitrile with 0.1% formic acid) were used throughout a 10 min run. The bioconjugates typically eluted in the first 3 min and were directed to ESI-qTOF MS (qTOFMicro, Waters Corporation). The valve was switched after 3 min to divert eluent to waste. The temperature was maintained at 100 °C for the ESI source and 250 °C for desolvation gas. The m/z scan range was from 500 to 4000 amu with the cone voltage set at 70 V. All solvents were purchased from Sigma-Aldrich except formic acid, which was from Thermo Scientific.

Immobilized Chymotrypsin Digestion. Before digestion, excess free pharmacophore-linker in all the conjugates was removed by 50K MWCO Zeba size exclusion spin columns (Thermo Scientific) according to the manufacture's instruction. The concentration of each bioconjugate was measured from the A280 absorbance by NanoDrop (Thermo Scientific), using an extinction coefficient of 1.47 mg/mL·cm. The protein concentration of all the bioconjugates and CVX-2000 was first adjusted to 16.0 mg/mL by dilution with the digestion buffer (50 mM Tris.HCl, 5 mM $CaCl_2$, pH 8.0). Then, the digestion was performed in the following order: (1) denaturation of the sample with 6 M guanadine-HCl at 37 °C for 30 min; (2) reduction with 10 mM dithiothreitol at 37 °C for 30 min; (3) alkylation with 15 mM iodoacetamide in the dark for 20 min; (4) dilution at 1:4 with the digestion buffer to reduce the denaturant concentration to 1.2 M; (5) addition of the immobilized chymotrypsin gel (Princeton Separations) at a 25:1 (w/w) substrate to enzyme ratio at 30 °C for 4 h at 300 rpm; (6) collection of the supernatant by centrifuging at 10 000 g for 5 min; (7) storage of the digest reactions at −20 °C until future use.

Immuno-Affinity Capture Coupled to MALDI-TOF MS (SELDI and MSIA). A mouse mAb was raised against the pharmacophore by using standard hybridoma techniques. The mAb was purified from ascites fluid using Protein A column and was dialyzed against phosphate buffer (pH 7.2; 0.02 M potassium phosphate, 0.15 M sodium chloride). For the SELDI assay, the mAb was first immobilized on a RS300 ProteinChip array (Bio-Rad). A volume of 5 μL was applied in each step unless specified otherwise. The mAb solution was diluted with PBS to 0.5 mg/mL and was added to each spot, and the array was incubated in a humid chamber overnight in a 4 °C fridge. All of the following experiments were then performed at room temperature. After removing the excess solution, each spot was washed with PBS twice and blocked with 1.0 mg/mL BSA in PBS for 2 h. After blocking, 0.1% CHAPS was added to rinse each spot twice. Then, each individual sample was added to each spot and the array was incubated for 2 h. The excess sample was then removed, washed sequentially by 0.1% CHAPS, PBS, and 5 mM HEPES for 30 s, and air-dried. A 0.5 μL of the saturated matrix solution composed of α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics) in 30% ACN with 1% TFA was applied to each spot twice. The array was air-dried, assembled to the MTP Bio-Rad 3 Chip Adaptor (Bruker Daltonics), and then analyzed by a reflectron AutoFlex Speed MALDI-TOF MS instrument (Bruker Daltonics).

dx.doi.org/10.1021/bc300413c | Bioconjugate Chem. 2013, 24, 53−62

For the MSIA assay, the MSIA affinity tips (Thermo Scientific) were preimmobilized with the same mAb reagent used for SELDI. Two electronic pipettes (Xplore, Eppendorf) were used in parallel by setting the programmed volume to 100 μL. Before binding, the tips were first equilibrated with 20 mM Tris-HCl pH 7.5 twice. Individual samples were then pipetted through the tips repetitively for 100 aspiration and dispensing cycles. After binding, the tip was rinsed sequentially by 0.1% CHAPS and distilled water for 10 cycles. The same CHCA matrix solution used for SELDI was spiked with a synthetic peptide (MW: 1658 Da, Sigma-Aldrich) as an internal standard (IS) at a final concentration of 0.6 ng/μL. The captured peptides from each sample were eluted with 5 μL of matrix solution as described above from each tip directly onto a spot in the MTP 384 ground steel MALDI plate (Bruker Daltonics).

MALDI-TOF MS Analysis and TOF/TOF Sequencing. The MALDI-TOF MS was operated by the *FlexControl* software (version 3.3) in positive mode for PMF analysis of the CVX-2000 digests, and the captured conjugated peptides from the SELDI and the MSIA assays. Each mass spectrum was acquired by summing 2000 laser shots in five different areas for each spot from m/z 500−3000 and externally calibrated with a mix of nine peptide standards (Peptide Calibration Standards 2, Bruker Daltonics) covering this mass range. The laser power was held constant between different samples for the SELDI and MSIA assay. Other major MS settings include 130 ns for pulse ion extraction, m/z 500 for deflection, and 2 GS/s for sample rate. *FlexAnalysis* (version 3.3) was used to label the peaks based on the built-in SNAP peak detection algorithm with an acceptable signal-to-noise ratio of >6 after smoothing and background subtraction. A mass tolerance of 100 and 200 ppm was used for the detection on the MALDI and the SELDI plate in PMF analysis. MS2 sequencing was performed by an UltraFlex TOF/TOF MS instrument (Bruker Daltonics) on the peaks representing potential conjugated peptides detected from the SELDI and the MSIA assay. The digested samples were desalted by C18 ziptip (Millipore) according to the manufacture's instruction and spotted on the MALDI plate for sequencing. Each selected precursor ion was fragmented separately by both laser-induced dissociation (LID) and collision-induced dissociation (CID). The fragments were separated from the precursor ion by the LIFT cell with the voltage set at 19 kV and the reflector voltage at 29.5 kV. The *S/N* threshold was set to 3, and a 0.5 Da mass tolerance was used for finding a match by comparing the experimental and theoretical spectrum.

RESULTS

Intact Mass Measurement of Bioconjugates by SEC-qTOF MS. It is important to evaluate the degree of conjugation under each reaction condition from a global view. The intact mass measurement was performed on deglycosylated samples which reduced the spectra heterogeneity caused by the presence of different N-linked glycoforms. Each sample containing a mixture of different conjugated species was analyzed by SEC-qTOF MS, and the weighted average number of conjugation additions (CA) was measured from the deconvoluted mass spectrum. A representative conjugation profile from the AZD and the NHS conjugate at 4:1 molar ratio is illustrated in Figure 2a. The NHS pharmacophore-linker bioconjugate distribution range spans from 0 CA, which represents the unconjugated Ab, to 5 CA with an interval of 1120 Da between adjacent peaks. This mass difference

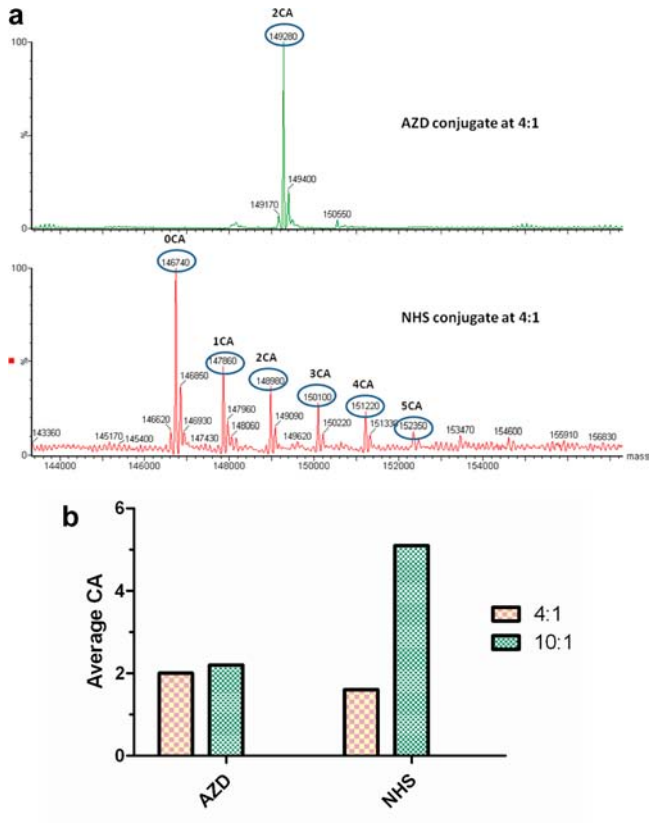


Figure 2. (a) Example of deconvoluted intact mass measurement spectra by SEC-qTOF MS. (b) Summary of average conjugate additions (CA) by using AZD and NHS linkers at 4:1 and 10:1 pharmacophore to Ab molar ratios.

corresponds to the addition of one NHS-linked peptide and is within ±5 Da error tolerance compared to the theoretically calculated mass of 1122 Da. The average CA was then calculated by integrating the intensity of each peak, and the results were summarized in Figure 2b for all four bioconjugates. For the reaction performed at a 4:1 molar ratio, the average CA is measured to be 2.0 and 1.6 for the AZD and NHS samples, respectively. Only a single species corresponding to 2 CA was detected in the deconvoluted spectrum of the AZD bioconjugates, while the NHS bioconjugates exhibit a broad distribution pattern, which is typical for non-site-specific conjugations. For the conjugation performed at a 10:1 molar ratio, the 3 CA species appear at low abundance in the AZD bioconjugates, which results in a slight increase of the average CA to 2.2, whereas the average CA has increased more obviously to 5.1 for the NHS bioconjugates.

MALDI-TOF MS Analysis of Immobilized Chymotrypsin Digests. The digestion efficiency was assessed prior to the assay development. The chymotryptic digests of the Ab scaffold (CVX-2000) was used for PMF analysis by MALDI-TOF MS. None of the conjugated samples were selected for this examination, because the risk of having false positives/negatives is higher in these samples without knowing the exact residue on which the conjugation may happen. For CVX-2000, the sequence coverage based on the percentage of amino acids is 63% and 58% for the HC and the LC, respectively, by applying a mass tolerance cutoff of 100 ppm. The coverage becomes slightly lower to 47% for the HC and 53% for the LC when the cutoff threshold is narrowed down to 50 ppm. These numbers

dx.doi.org/10.1021/bc300413c | *Bioconjugate Chem.* 2013, 24, 53−62

are above previous studies, which reported ~30% sequence coverage observed within 50−100 ppm mass tolerance for a single protein after chymotrypsin digestion,[27,28] and is consistent with general digestion which often yields 20−60% coverage by using MALDI-TOF MS. To be noted, the choice of chymotrypsin is beneficial for the MALDI-TOF MS detection of conjugated peptides, which are mainly from 1500 to 3000 Da, whereas tryptic peptides are much larger especially if the Lys is modified by the pharmacophore-linker.

Assay Development by Using the Site-Specific Conjugation Model System. The proof-of-concept phase was conducted by using the AZD conjugates at 4:1 molar ratio, because this sample represents a well-known site-specific conjugation model. After digestion, 1 μL of the peptide mixture corresponding to a total amount of 0.8 μg was used for each iteration of SELDI and MSIA experiments to optimize the conditions. Figure 3 visualizes a comparison of three MALDI-

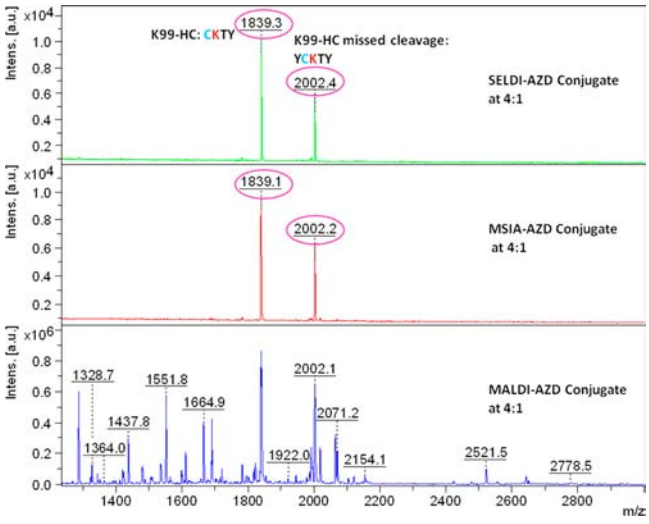


Figure 3. Comparison of spectra before (MALDI) and after immuno-capture (SELDI and MSIA) by using AZD conjugate at 4:1 pharmacophore+linker to Ab molar ratio.

TOF MS spectra obtained from capturing the conjugated peptides by SELDI, MSIA, and no capture. Only two major peaks are shown in both SELDI and MSIA spectra, while nearly 100 peaks are detected from the same digests without capture. This demonstrates that the utilization of immuno-affinity capture coupled to MALDI-TOF can greatly reduce spectrum complexity compared to the direct spotting method.

In order to determine the sites of conjugation from the MS spectra, a straightforward strategy has been designed to interpret the peaks by leveraging our prior knowledge that the pharmacophore with the AZD linker has a high specificity toward a particular Lys on each HC of CVX-2000. In practice, an in silico chymotrypsin digestion of CVX-2000 was simulated to create a database containing only the sequence information about the HC and the LC peptides. The mass addition of the AZD-linked pharmacophore was specified as a variable modification to Lys residues only. Then, the m/z of captured peptides was searched against the database by MASCOT. Searching scores indicating statistical significance were not taken into consideration in this study, because the purpose is just to find a match by PMF within the specified mass tolerance range, instead of performing a large-scale proteome profiling. Also, less stringent mass tolerance is allowed for SELDI. This is because the fixed distance between each spot in a SELDI array is larger than the standard MALDI plate, which compromises the inherent mass accuracy after external calibration. Two hits have been obtained within 200 ppm for the SELDI spectrum and 100 ppm for the MSIA spectrum. The primary peak (m/z: 1839.0 ± 0.3) has been matched to a scaffold peptide ($C^{98}K^{99}T^{100}Y^{101}$) and the secondary peak (m/z: 2002.1 ± 0.2) has been matched to a miss-cleavage variant ($Y^{97}C^{98}K^{99}T^{100}Y^{101}$) of the same peptide. This is consistent with our knowledge that the pharmacophore with AZD linker is fused specifically to K99 on each HC when a molar ratio 3:1 is used in the reaction.[29]

Identification of Heterogeneous Conjugation Sites. The established SELDI and MSIA assays were then applied to identify unknown conjugation sites. To compare the sensitivity of these two assays for detecting complex analytes, digests from the two conjugates at a 10:1 molar ratio were used because these samples contain more heterogeneous conjugation species than the samples conjugated at 4:1 molar ratio. Figure 4 shows

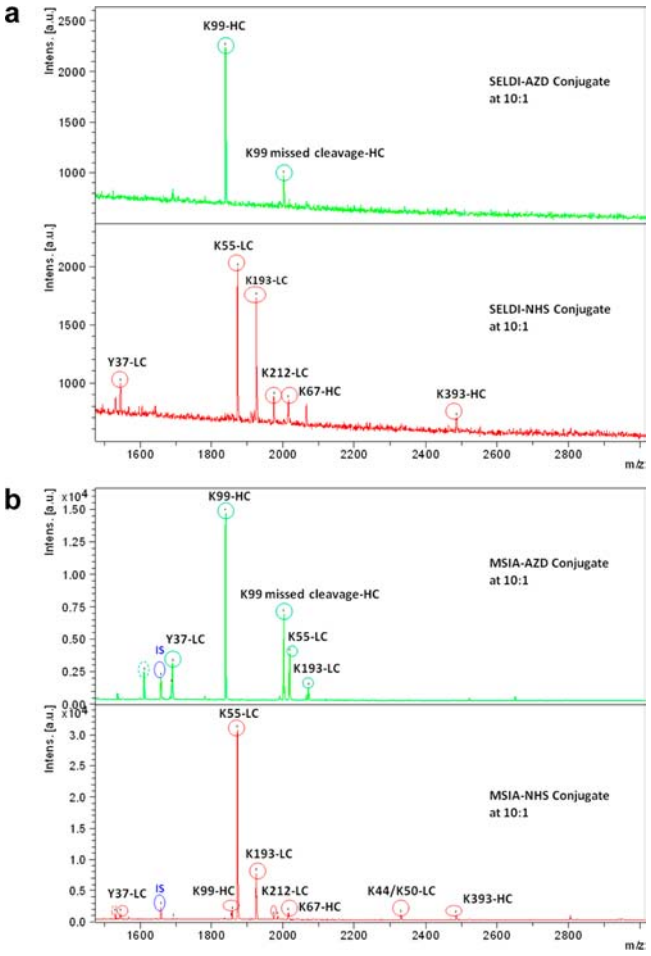


Figure 4. (a) Comparison of captured AZD and NHS conjugated peptides by SELDI. (b) Comparison of captured AZD and NHS conjugated peptides by MSIA.

the captured peptide results from SELDI (a) and MSIA (b) by loading the same amount of sample (1 μL, 0.8 μg). For the SELDI assay, the same two peptides described above were captured in the AZD conjugate, and multiple peaks were detected in the NHS conjugate. For the MSIA assay, the spectrum generated from each type of conjugate is more

dx.doi.org/10.1021/bc300413c | Bioconjugate Chem. 2013, 24, 53−62

complicated than the corresponding SELDI spectrum representing potentially more conjugated peptides captured.

A mixed scheme has been employed to decipher unknown conjugation sites. It contains a sequential workflow as follows: (1) perform the aforementioned straightforward MASCOT search by only allowing Lys as the modifiable residue; (2) adopt a backward searching strategy for the rest of unmatched peaks. Specifically, the mass addition of the AZD/NHS linked pharmacophore was subtracted from the detected monoisotopic mass. The remaining mass of each peak which represents the unconjugated peptide is then used for mass mapping to find if there is a match to any chymotryptic peptide of the Ab scaffold. This allows for the inference of any new conjugation site and is not confined to Lys. TOF/TOF MS sequencing data are then examined to locate the exact residue. To be noted, the pharmacophore used in this study does not contain any chymotrypsin cleavage sites; thus, the detected mass of each peak can be directly used for the calculation. A similar strategy working backward to deduce amino acid sequence containing the conjugation site has been adopted in other studies as well.[30] (3) Optionally, refine the MASCOT searching criteria to expand the variable modification of the pharmacophore-linker to additional residues identified from step 2. The final parameter setting can be generalized to perform a straightforward MASCOT search on similar conjugates. This mixed scheme combines the utilization of prior knowledge and a learning process to quickly identify unknown conjugation sites with minimized false positives.

Peaks that are assigned to conjugated peptides are circled and annotated in Figure 4. Detailed sequence information for each corresponding peak is also summarized in SI Table 1S. Six peaks are detected in the SELDI spectrum for the NHS conjugate with the majority being located on the LC. The primary peak (m/z: 1872.1 ± 0.3) has been matched to a peptide ($K^{55}V^{56}S^{57}N^{58}R^{59}F^{60}$). The secondary peak ($m/z$: 1925.1 ± 0.3) with lower intensity has been matched to another peptide ($E^{192}K^{193}H^{194}K^{195}V^{196}Y^{197}$). The third peak ($m/z$: 1544.9 ± 0.3) was not able to be assigned using the straightforward strategy. However, the backward search identifies a match to a four amino acid peptide ($G^{34}S^{35}P^{36}Y^{37}$), and MS2 sequencing, which will be discussed later, confirms the peptide identity and locates the conjugation site to Tyr. The spectra obtained from the MSIA assay (Figure 4b) by using the same samples are more complicated than the SELDI spectra, such that extra peaks are detected in addition to all the ones captured by SELDI. Each of the MSIA spectra also contains an extra peak at m/z: 1657.7 which is the IS spiked into the elution matrix. The intensities of the additional sites detected in MSIA only are all below the signal from the sites detected by both two methods, suggesting that MSIA is more amenable for detection of low-abundance conjugation species in our study. Also, the absolute intensity shown in the *Y*-axis based on the most abundant peak in all MSIA spectra is 5-fold higher than in the SELDI spectra. This together with the detection of lower intensity peaks indicates the MSIA assay provides a wider dynamic range for detection.

MS2 Sequencing to Confirm Sites of Conjugation. Targeted tandem MS has been performed to confirm the identity of the scaffold peptide and the sites of conjugation. MS2 sequencing can be skipped in some scenarios (i.e., the conjugation happens on a particular residue exclusively) and for other application purpose (i.e., distinguishing between adjacent sites is not required), especially with the help of LC coupled high resolution MS1 spectra (<2 ppm) for accurate mass measurement. However, MS2 sequencing is necessary under any of the following three conditions in our study: (1) two candidate scaffold peptides with similar mass (less than 0.5 Da) are present; (2) more than one Lys is present in the scaffold peptide; (3) other residues (Tyr, Ser, and Thr) which have the possibility of forming ester bond with all two linkers are present in the absence of Lys. In addition, MALDI-TOF MS has relatively low resolution compared to ESI-qTOF MS, although 50% of the identifications are matched within 20 ppm in our study to evaluate digestion efficiency. Thus, it is very beneficial to employ MS2 sequencing in our case to accurately identify the sites of conjugation.

TOF/TOF MS implemented by the "LIFT" technology in UltraFlex (Bruker Daltonics) has been employed to sequence every suspicious peak detected in the SELDI and MSIA spectra. MS2 spectra were acquired separately under both LID and CID modes, and very similar fragmentation patterns were observed, except that more immonium ions were detected under CID. The same finding by using the same instrument was published previously.[31] Figure 5 illustrates a representative LID MS2 spectrum by isolating the peak (m/z 1925.1 ± 0.3) in the NHS conjugate as the precursor ion. The overall structure of this branched peptide is displayed on the top in the same figure. Although LID produces relatively cleaner spectra than CID, massive peaks are present in the MS2 spectrum resulting from different types of cleavages. In practice, this branched peptide was entered as two separate chains and then linked between the N-terminal residue of the pharmacophore, and either the first or the second Lys in EKHKVY by using *SequenceEditor* (Bruker Daltonics). Then, the theoretical spectrum simulated from this branched peptide is applied back to match the experimental spectrum by using a mass tolerance of 0.5 Da. This is implemented by *BioTools 3.2* (Bruker Daltonics) which visualizes the results as a set of two sequences. The cross-linked sequence is treated as a modification to the other selected chain. In Figure 5, the pharmacophore is selected as

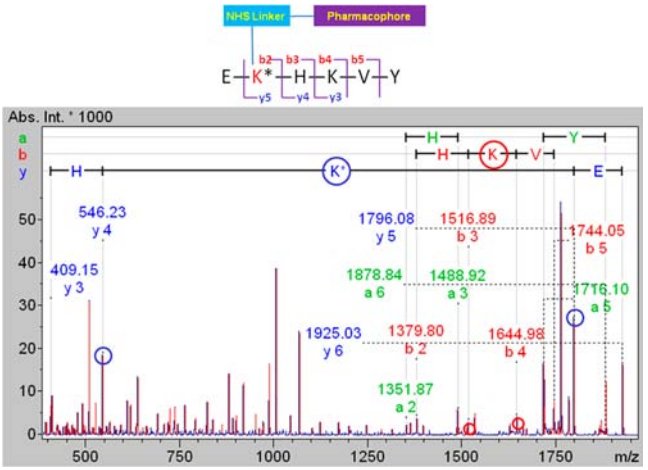


Figure 5. Example of MS2 spectra to confirm the conjugation site. The overall structure of a conjugated peptide EK*HKVY with observed backbone fragmentation is displayed on the top. This branched peptide can be viewed as two separate peptides with respective chain treated as a modification to the other one. The annotated MS2 spectra are generated by treating the pharmacophore as a modification to the Ab scaffold peptide. The detection of b and y-ion series locates the conjugation to the first Lys residue unambiguously.

dx.doi.org/10.1021/bc300413c | *Bioconjugate Chem.* 2013, 24, 53−62

the modification to the scaffold peptide in order to annotate the peaks matched to the EKHKVY fragments. The peptide identity is verified, and more importantly, the detection of y4 and y5 ions locates the conjugation site to the first Lys (circled in blue); the detection of b3 and b4 ions rules out the second Lys (circled in red).

Relative Quantification by the MSIA Assay. The MSIA assay has been demonstrated in Figure 4 to have higher sensitivity to detect more low-abundance conjugation species which cannot be detected in SELDI, and the response of targeted analytes in MSIA has been found to behave proportionally to sample concentration, whereas there is no such a linear pattern observed in SELDI. A serial dilution at nine concentration points has been performed to further investigate the dynamic range of the MSIA assay. For each bioconjugate, a 100 μL digested sample with the starting concentration at 800 μg/mL was diluted to 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 μg/mL. Only the concentration of the total digests was referred to here, as the fraction of the analytes is unknown. In order to evaluate assay reproducibility, the whole MSIA experimental procedure has been repeated three times at each dilution point by using 100 μL NHS conjugate at 10:1 molar ratio as the example. MALDI-TOF MS is not intrinsically quantitative; however, it can achieve relative quantification for the same analyte across different samples by spiking an IS. The use of IS compensates for the signal variations which we believe are caused by the nonuniform matrix-analyte crystal formation and incomplete sampling to cover the whole spotted area during data acquisition. A commercially available synthetic peptide (m/z: 1657.7) was selected as the IS because it has no mass interference with the targeted peaks. The concentration of the IS has also been optimized so that the intensity detected from the IS is on the lower end of analyte response range to minimize ion suppression, and the optimal amount to spike into the matrix has been determined to be at a final concentration of 0.6 ng/μL. The intensities from the top two conjugation sites (K155-LC and K193-LC) in the NHS bioconjugates have been extracted, normalized by the IS, and plotted against the corresponding concentration of the total digest. Figure 6 shows that the linear response ranges from 62.5 ng/mL (12 800× dilution) to 8.0 μg/mL (100× dilution) and it plateaus at about 8.0 μg/mL for both targets, indicating a dynamic range of 128-fold. The average CV across all concentration points is 11% for the primary site K55-LC and 44% for the secondary site K193-LC in the NHS conjugates. Variations contributing to the final CV value are from two sources: affinity capturing and MALDI-TOF MS detection. Analytes at lower abundance are more susceptible to competitive binding when multiple antigens (Ag) are present in the capturing process. Moreover, they are also more vulnerable to ion suppression, and the degree of influence may not be consistent between different laser shots. Therefore, a relatively higher CV has been observed for the secondary site. Nevertheless, the goodness of fit is calculated to be 0.98 and 0.96 for the primary and secondary site, respectively. Also, the dynamic range not only indicates a linear response range, but also aligns well with an appropriate sample loading range to minimize unspecific binding. When the samples was loaded at 80.0 μg/mL (10× dilution), an extra peak representing an unconjugated scaffold peptide resulting from unspecific binding appears, whereas the same peak is barely detectable at 100× dilution. The same serial dilution has also been performed in the AZD conjugate at 10:1 molar ratio and similar dynamic range has been observed (Figure 2S). The sensitivity and linearity results together provide us information about the suitable sample concentration and enable relative quantification to evaluate conjugation pattern for analytes within the linear range.

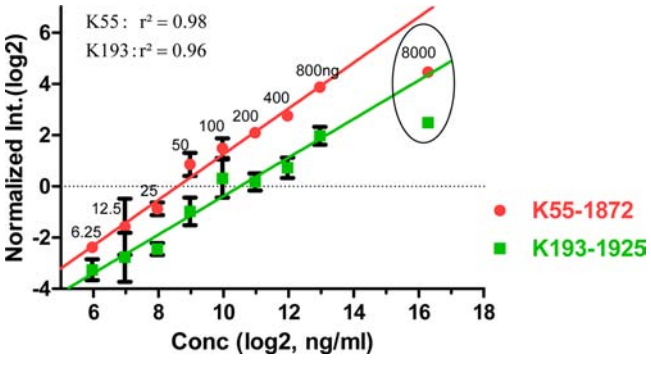


Figure 6. MSIA dynamic range for two captured NHS conjugated peptides at 10:1 molar ratio. Normalized intensities extracted from each peptide are plotted against the concentration of whole digests on a log2 scale. The corresponding total loading amount is displayed adjacent to each point in nanograms. The linear range spans from 6.25 to 800 ng for both peptides with the goodness of fit being 0.98 and 0.96, respectively. The most concentrated samples (circled) for both peptides are significantly deviated from the regression lines, indicating that the work range plateaus at the total amount of 800 ng.

Practical Application as a Stability Indicating Assay. Once the sites of conjugation have been determined, direct PMF analysis of the conjugates by MALDI-TOF MS can be used as a stability indicating assay based on the known m/z of targeted peaks. A 4-week stability study was performed on the intact NHS bioconjugates at 10:1 molar ratio stressed at 40 °C. The stacked spectra in Figure 3S show that the signal from the peak at m/z 1544.9 representing the $G^{34}S^{35}P^{36}Y^{37}$ peptide decreased significantly after one week and became undetectable after two weeks. In contrast, the signal level of an unconjugated peptide at m/z 1551.8 remains the same. These results indicate that the NHS conjugation on Lys residue via the amide bond is more stable than the conjugation on Tyr residue via the ester bond at 40 °C. Similar assays can be applied to study bioconjugate stabilities under other stressed conditions.

DISCUSSION

Simplification by Immuno-Affinity Capture. This study demonstrates the use of immuno-affinity capture as a stringent filter to greatly reduce spectra complexity and allow for the majority of peaks shown in the MS spectra to be conjugated peptides. This simplification strategy takes advantage of the sequence in the phamacophore that there is no enzymatic cleavage site in the epitope for Ab binding. If the pharmacophore is susceptible to enzyme cleavage in the digestion process, then it is important to evaluate if the specific epitope would be disrupted to impair binding. This method is also applicable to identify sites of conjugation by toxins which commonly do not contain enzyme cleavage sites.

Advantages of MSIA over SELDI in Our Application. *Lower Limit of Detection and Wider Dynamic Range.* The MSIA assay has been demonstrated to have the capability to capture more low-abundance conjugated species than the SELDI assay. The reason MSIA is more sensitive is probably its higher surface capacity to covalently attach more Ab. Thus,

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more Ab may be available to capture Ag in the tip format than the plate format, although it is difficult to quantify the total amount of Ab immobilized in each format. Linearity is another important feature that we have observed in MSIA but not in SELDI. The abilities of MSIA to enable homogeneous docking of Ag during the binding process and uniform matrix-analyte crystal formation on the MALDI plate could be essential to its possession of a wide range of linear response. Specifically, two steps of the MSIA assay may facilitate this feature: (1) Extensive aspiration−dispense cycles are conducted to enable a larger amount of material to contact the Ab. (2) Premixing of the matrix and analytes is also performed simultaneously when captured peptides are eluted directly on the MALDI plate. Conversely, the capability to enable agitation during the Ab and Ag binding process is restricted in the SELDI assay due to the plate format. Thus, the analytes could be clustered in a certain area and totally absent in the neighboring area in the same spot on the SELDI plate. In addition, it is not possible to premix the analytes with the matrix, as the analytes have already been dried on each spot. Although the spotting of matrix has been repeated twice, the crystal formation may still not be uniform and consequently adds additional variation. As a result, the integrated signal from the SELDI assay is not detected reproducibly enough to exhibit a linear response in our study.

Higher Throughput. The overall sample processing time after digestion and before detection is about 15 min per sample for the MSIA assay and about one day for the SELDI assay, although multiple samples can be processed together in the latter case. An overnight incubation time is needed in SELDI to attach the Ab, and each step in the subsequent capturing process needs one or two hours, whereas the MSIA tips have already been immobilized with the Ab of choice and the binding can be accomplished much more rapidly. From a practical point of view, SELDI may be more suitable for use as an exploratory tool for initial discovery and MSIA can be used for follow-up investigations. Also, the throughput of MSIA assay can be further improved by using a liquid handling robot to automate the current experimental protocol. A multiplexed MSIA assay based on the current workflow is under development to analyze in vivo samples.

Identification of the Tyr Conjugation Site. It is always beneficial to use an orthogonal MS platform to validate an identification when sequence ambiguity is encountered. For example, the identification of the Tyr conjugated peptide ($G^{34}S^{35}P^{36}Y^{37}$) on the LC has posed a potential uncertainty mainly arising from the presence of another chymotryptic scaffold peptide ($M^{34}S^{35}W^{36}$) on the HC with a mass difference of 0.01 Da compared to $G^{34}S^{35}P^{36}Y^{37}$. Resolving such a slight difference requires both the mass accuracy above 6.5 ppm and the resolution above 150k which cannot be achieved by current MALDI-TOF MS. Moreover, the presence of Ser in the peptide further complicates the issue, as theoretically Ser could also be conjugated to form the same ester bond. Although the MS2 spectra from TOF/TOF resolves the problem, results from accurate mass measurement with 0.5 ppm from LC-MS further confirm the finding by using an Acquity UPLC coupled to Synapt G2 HDMS system with lock spray(data not shown). Interestingly, this Tyr conjugation site is located in the C-terminus which represents a special scenario in that the enzymatic cleavage can still occur with this modification. This may be due to no change in the hydrophobicity in the catalytic pocket of chymotrypsin. Moreover, this Tyr residue exhibits an unusually higher reactivity, even when compared to the majority of primary amines from Lys side chains except for those aforementioned predominant Lys sites. A possible explanation for this is the surrounding three-dimensional environment and may be responsible for facilitating its solvent accessibility.

Comparison of Linker Site-Specificity between AZD vs NHS. Because the mass of each conjugated peptide has been determined from SELDI and MSIA, a direct MALDI-TOF PMF analysis was performed to compare the conjugation distribution between the AZD and NHS conjugates. The same epitope is embedded in the pharmacopore; however, the immobilized Ab may still have different binding affinities toward different conjugated peptides depending on the physical/chemical properties of the additional portion (the scaffold peptide). Therefore, in order to avoid the variation resulting from different assay formats and the immuno-affinity capture process itself, a direct MALDI-TOF analysis was employed as a more straightforward comparison strategy for the AZD and the NHS conjugates after desalting by C18 zip-tips. The conjugate at a 4:1 molar ratio for each linker was selected because this condition is closer to what is used in practical development. The signal intensities from the top four most abundant conjugation sites were extracted and normalized against the most abundant site. A different conjugation distribution pattern is observed between the AZD and the NHS bioconjugates as shown in SI Figure 4S. The AZD bioconjugate shows a predominant site (K99-HC), whereas the NHS conjugate exhibits a more even distribution among the four conjugation sites. To be noted, only relative comparison between the AZD and NHS conjugates is feasible in our study. Comparison of site occupancy between different conjugation sites requires simultaneous monitoring of the signals from the conjugated peptide and the same unconjugated peptide, as well as corresponding reference standard to eliminate the error caused by different ionization efficiency. However, it is difficult to detect the unconjugated form by MALDI-TOF MS because chymotrypsin digestion typically generates fragments about 600 Da, which is in the low mass detection range where matrix interference is significant.

■ CONCLUSION

In this paper, we have demonstrated a novel application by coupling immuno-affinity capture to MALDI-TOF MS to quickly identify bioconjugation sites. A mAb reagent against the pharmacophore was utilized to capture the conjugated peptides after immobilized chymotrypsin digestion. An AZD linked bioconjugate was first used as a model system to develop the assay, and then the established workflow was applied to identify NHS conjugation sites under two different reaction conditions. Multiple conjugation sites have been rapidly identified for each linker under each specific condition and confirmed by MS2 sequencing afterward, such as K99 on the HC and K55 on the LC shown to be the primary sites for the AZD and NHS linkers, respectively. We have also investigated two different capture formats by comparing SELDI vs MSIA tips. The MSIA assay has exhibited higher sensitivity and linearity over a broad dynamic range from relative quantification measurement. Furthermore, we have evaluated the conjugation pattern between the AZD and NHS linked bioconjugates which leads to a conclusion that NHS exhibits a more even distribution than AZD. Also, another application as a stability indicating assay to study labile conjugation sites has been illustrated based on the acquired sequence information. This workflow can be

dx.doi.org/10.1021/bc300413c | *Bioconjugate Chem.* 2013, 24, 53−62

further modified as a multiplexed assay platform by automating the whole process. The application can also be extended to analyze in vivo samples by reversing the capture format (i.e., reagent against the conjugated scaffold peptides is immobilized instead) for further characterization of in vivo pharmacophore/drug modifications. Overall, this study reports a general workflow to quickly identify sites of conjugation and can be applied to study any combination of different linkers and Ab scaffolds.

■ ASSOCIATED CONTENT

Supporting Information

Additional graphics and data table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank our colleague Anna Tempczyk for help with the crystallographic graph and Venkata Doppalapudi for the preparation of the pharmacophore-linker reagents.

■ REFERENCES

(1) Shen, B.-Q., Xu, K., Liu, L., Raab, H., Bhakta, S., Kenrick, M., Parsons-Reponte, K. L., Tien, J., Yu, S.-F., Mai, E., Li, D., Tibbitts, J., Baudys, J., Saad, O. M., Scales, S. J., McDonald, P. J., Hass, P. E., Eigenbrot, C., Nguyen, T., Solis, W. A., Fuji, R. N., Flagella, K. M., Patel, D., Spencer, S. D., Khawli, L. A., Ebens, A., Wong, W. L., Vandlen, R., Kaur, S., Sliwkowski, M. X., Scheller, R. H., Polakis, P., and Junutula, J. R. (2012) Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat. Biotechnol. 30*, 184−189.

(2) Junutula, J. R., Raab, H., Clark, S., Bhakta, S., Leipold, D. D., Weir, S., Chen, Y., Simpson, M., Tsai, S. P., Dennis, M. S., Lu, Y., Meng, Y. G., Ng, C., Yang, J., Lee, C. C., Duenas, E., Gorrell, J., Katta, V., Kim, A., McDorman, K., aFlagella, K., Venook, R., Ross, S., Spencer, S. D., Wong, W. L., Lowman, H. B., Vandlen, R., Sliwkowski, M. X., Scheller, R. H., Polakis, P., and Mallet, W. (2008) Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat. Biotechnol. 26*, 952−932.

(3) Hermanson, G. T. (2008) *Bioconjugate Techniques*, 2nd ed., Academic Press Inc., New York.

(4) Doppalapudi, V. R., Tryder, N., Li, L., Aja, Teresa, Griffith, D., Liao, F.-F., Roxas, G., Ramprasad, M. P., Bradshaw, C., and Carlos F. Barbas, I. (2007) Chemically programmed antibodies:Endothelin receptor targeting CovX-Bodies™. *Bioorg. Med. Chem. Lett. 17*, 501−506.

(5) Doppalapudi, V. R., Huang, J., Liu, D., Jin, P., Liu, B., Lia, L., Desharnais, J., Hagen, C., Levin, N. J., Shields, M. J., Parish, M., Murphy, R. E., Rosario, J. D., Oates, B. D., Lai, J.-Y., Matina, M. J., Ainekulu, Z., Bhat, A., Bradshaw, C. W., Woodnutt, G., Lerner, R. A., and Lappe, R. W. (2010) Chemical generation of bispecific antibodies. *Proc. Natl. Acad. Sci. U. S. A. 107*, 22611−22616.

(6) Kalkhof, S., and Sinz, A. (2008) Chances and pitfalls of chemical cross-linking with amine-reactive N-hydroxysuccinimide esters. *Anal. Bioanal. Chem. 392*, 305−312.

(7) Wang, L., Amphlett, G., Blättler, W. A., Lambert, J. M., and Zhang, W. (2005) Structural characterization of the maytansinoid−monoclonal antibody immunoconjugate, huN901−DM1, by mass spectrometry. *Protein Sci. 14*, 2436−2446.

(8) Manikwar, P., Zimmerman, T., Blanco, F. J., Williams, T. D., and Siahaan, T. J. (2011) Rapid identification of fluorochrome modification sites in proteins by LC ESI-Q-TOF mass spectrometry. *Bioconjugate Chem. 22*, 1330−1336.

(9) Iglesias, A. H., Santos, L. F. A., and Gozzo, F. b. C. (2010) Identification of cross-linked peptides by high-resolution precursor ion scan. *Anal. Chem. 82*, 909−916.

(10) Murphy, R. E., Kinhikar, A. G., Shields, M. J., Rosario, J. D., Preston, R., Levin, N., and Ward, G. H. (2010) Combined use of immunoassay and two-dimensional liquid chromatography mass spectrometry for the detection and identification of metabolites from biotherapeutic pharmacokinetic samples. *J. Pharm. Biomed. Anal. 53*, 221−227.

(11) Xu, K., Liu, L., Saad, O. M., Baudys, J., Williams, L., Leipold, D., Shen, B., Raab, H., Junutula, J. R., Kim, A., and Kaur, S. (2011) Characterization of intact antibody−drug conjugates from plasma/serum in vivo by affinity capture capillary liquid chromatography−mass spectrometry. *Anal. Biochem. 412*, 56−66.

(12) Liang, X., and Lubman, D. M. (1998) On-probe immunoaffinity extraction by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem. 70*, 498−503.

(13) Tang, N., Tornatore, P., and Weinberger, S. R. (2004) Current developments in SELDI affinity technology. *Mass Spectrom. Rev. 23*, 34−44.

(14) Seiberta, V., Wiesnerb, A., Buschmanna, T., and Meuer, J. o. (2004) Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI TOF-MS) and ProteinChip technology in proteomics research. *Pathol. Res. Pract. 200*, 83−94.

(15) Bencharit, S., and Al-Tarawneh , S. K. (2009) Applications of Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight (SELDI-TOF) Mass Spectrometry in Defining Salivary Proteomic Profiles. *Open Dent. J. 3*, 74−79.

(16) Villemagne, V. L., Perez, K. A., Pike, K. E., Kok, W. M., Rowe, C. C., White, A. R., Bourgeat, P., Salvado, O., Bedo, J., Hutton, C. A., Faux, N. G., Masters, C. L., and Barnham, K. J. (2010) Blood-borne amyloid-beta dimer correlates with clinical markers of Alzheimer's disease. *J. Neurosci. 30*, 6315−6322.

(17) Liu, J.-Y., Jin, L., Zhao, M.-Y., Zhang, X., Liu, C.-B., Zhang, Y.-X., Li, F.-J., Zhou, J.-M., Wang, H.-J., and Li, J.-C. (2011) New serum biomarkers for detection of tuberculosis using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. *Clin. Chem. Lab Med. 49*, 1727−1733.

(18) Nelson, R. W., Dogruel, D., Krone, J. R., and Williams, P. (1995) Peptide characterization using bioreactive mass spectrometer probe tips. *Rapid Commun. Mass Spectrom. 9*, 1380−1385.

(19) Nelson, R. W., Krone, J. R., Bieber, A. L., and Williams, P. (1995) Mass spectrometric immunoassay. *Anal. Chem. 67*, 1153−1158.

(20) Nelson, R. W., and Borges, C. R. (2011) Mass spectrometric immunoassay revisited. *J. Am. Soc. Mass Spectrom. 22*, 960−968.

(21) Niederkofler, E. E., Kiernan, U. A., O'Rear, J., Menon, S., Saghir, S., Protter, A. A., Nelson, R. W., and Schellenberger, U. (2008) Detection of endogenous B-type natriuretic peptide at very low concentrations in patients with heart failure. *Circ. Heart Fail. 1*, 258−264.

(22) Trenchevska, O., Kamcheva, E., and Nedelkov, D. (2010) Mass spectrometric immunoassay for quantitative determination of protein biomarker isoforms. *J Proteome Res. 9*, 5969−5973.

(23) Kiernan, U. A., Phillips, D. A., Trenchevska, O., and Nedelkov, D. (2011) Quantitative mass spectrometry evaluation of human retinol binding protein 4 and related variants. *PLoS One 6*, e17282.

(24) Trenchevska, O., Kamcheva, E., and Nedelkov, D. (2011) Mass spectrometric immunoassay for quantitative determination of transthyretin and its variants. *Proteomics 11*, 3633−3641.

(25) Wang, W. (2011) Identification of respective lysine donor and glutamine acceptor sites involved in factor XIIIa-catalyzed fibrin α chain cross-linking. *J. Biol. Chem. 286*, 44952−44964.

(26) Kühn-Hölsken, E., Lenz, C., Sander, B., Lührmann, R., and Urlaub, H. (2005) Complete MALDI-ToF MS analysis of cross-linked

peptide-RNA oligonucleotides derived from nonlabeled UV-irradiated ribonucleoprotein particles. *RNA 11*, 1915−1930.
(27) Fischer, F., and Poetsch, A. (2006) Protein cleavage strategies for an improved analysis of the membrane proteome. *Proteome Sci. 4*, 2−14.
(28) Locke, D., Bian, S., Li, H., and Harris, A. L. (2009) Post-translational modifications of connexin26 revealed by mass spectrometry. *Biochem. J. 424*, 385−398.
(29) Huang, H., Lai, J.-Y., Do, J., Liu, D., Li, L., Rosario, J. D., Doppalapudi, V. R., Pirie-Shepherd, S., Levin, N., Bradshaw, C., Woodnutt, G., Lappe, R., and Bhat, A. (2011) Specifically targeting angiopoietin-2 inhibits angiogenesis, tie2-expressing monocyte infiltration, and tumor growth. *Clin. Cancer Res. 17*, 1001−1011.
(30) Chiu, M. L., Ytterberg, A. J., Loo, R. R. O., Loo, J. A., and Monbouquette, H. G. (2011) Characterization of morphine-glucose-6-phosphate dehydrogenase conjugates by mass spectrometry. *Bioconjugate Chem. 22*, 1595−1640.
(31) Shenar, N., Sommerer, N., Martineza, J., and Enjalbala, C. (2009) Comparison of LID versus CID activation modes in tandemmass spectrometry of peptides. *J. Mass Spectrom. 44*, 621−632.
(32) Alley, S. C., Benjamin, D. R., Jeffrey, S. C., Okeley, N. M., Meyer, D. L., Sanderson, R. J., and Senter, P. D. (2008) Contribution of linker stability to the activities of anticancer immunoconjugates. *Bioconjugate Chem. 19*, 759−765.