Complete Characterization of a Cysteine-linked Antibody-Drug Conjugate Performed on a Hybrid Quadrupole-Orbitrap Mass Spectrometer with High Mass Range

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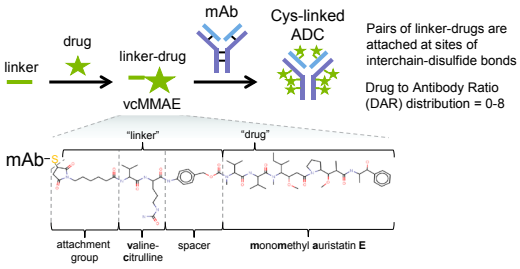
ABSTRACT

We have modified the instrument control software of a benchtop quadrupole-Orbitrap mass spectrometer to add native MS capability. In this study we demonstrate complete characterization of *Brentuximab vedotin*, a cysteine-linked ADC, which requires native MS conditions for intact analysis. We demonstrate preservation of non-covalent bonding of antibody subunits during electrospray ionization. HMR mode can be turned off for peptide mapping. We use trypsin peptide mapping approach with HCD fragmentation to achieve 99% coverage of the *Brentuximab vedotin* sequence using a single LC-MS analysis of a 90 min reverse phase gradient. Finally, we demonstrate that signature ions specific for HCD fragmentation of Brentuximab vedotin can be utilized to increase MS/MS assignment confidence.

INTRODUCTION

The complexity of modern therapeutic proteins, such as antibody-drug conjugates (ADCs), present a great analytical challenge which requires high resolution chromatography combined with high resolution mass spectrometry. Complementary MS approaches such as peptide mapping and intact mass analysis are needed for complete characterization of therapeutic proteins. Cysteine-linked ADCs present a unique challenge for characterization as proper intact analysis requires native MS conditions to preserve structurally-critical non-covalent binding between antibody chains. We have modified commercially-available Thermo Scientific™ Q Exactive™ Plus and Q Exactive™ HF Orbitrap™ mass spectrometers to perform native LC-MS experiments. In the present study, we demonstrate this capability with intact analysis of *Brentuximab vedotin*, a cysteine-linked ADC **(Figure 1)**. Additionally, we have performed denaturing LC-MS and peptide mapping on these same instruments to generate complementary datasets for complete characterization.

Figure 1. Schematic for Constructing Cysteine-Linked ADC
Brentuximab vedotin is a cysteine-linked ADC which is constructed by modifying an antibody with vcMMAE, a preformed linker-drug comprised of a valine-cirtuline-based linker and a monomethyl auristatin E toxic drug. Saturatured (8 drugs) cys-linked ADCs are held intact with only non-covalent binding.



MATERIALS AND METHODS

Brentuximab vedotin was prepared for peptide mapping (reduction, alkylation, and trypsin digestion) or intact analysis (no treatment). For denaturing LC-MS intact analysis 1 µg of protein samples were separated using a 10 min gradient of 10-90% ACN in H_2O and 0.1% formic acid (Thermo MAb-Pac RP; flow rate 250 µL/min). For native LC-MS intact analysis 10 µg of sample was desalted online using size exclusion chromatography (Waters™ BEH SEC 4.6x150mm; 50 mM NH_4OAc isocratic elution, flow rate 300 µL/min) and directly presented to the mass spectrometer via electrospray ionization. Peptide mapping was performed using 2.5 µg of sample separated using a 90 min gradient of 2-90% ACN in H_2O and 0.1% formic acid (Acclaim RSLC 120 C18; flow rate 250 µL/min). Commercially-available Orbitrap mass spectrometers (Q Exactive HF and Q Exactive Plus) which were modified to include High Mass Range (HMR) mode to allow improved high mass transmission and scanning up to m/z 8000. Native intact and denaturing MS data were acquired in HMR mode at setting of R=15k or 17.5k and deconvolved using the ReSpect™ algorithm and Sliding Window integration in Thermo Scientific™ BioPharma Finder™ 1.0 SP1 software. Deconvolution species were identified automatically using the publicly-available FASTA sequence for Brentuximab vedotin, a mass tolerance of 50 ppm, and a static modification of Gln>Pyro-Glu for the heavy chain. Peptide mapping data were acquired by data dependent selection with R=60k or 70k for Full MS and R=15k or 17.5k for MS/MS. Peptide mapping data were searched using the MassAnalyzer algorithm in BioPharma Finder software with a tolerance of 5 ppm.

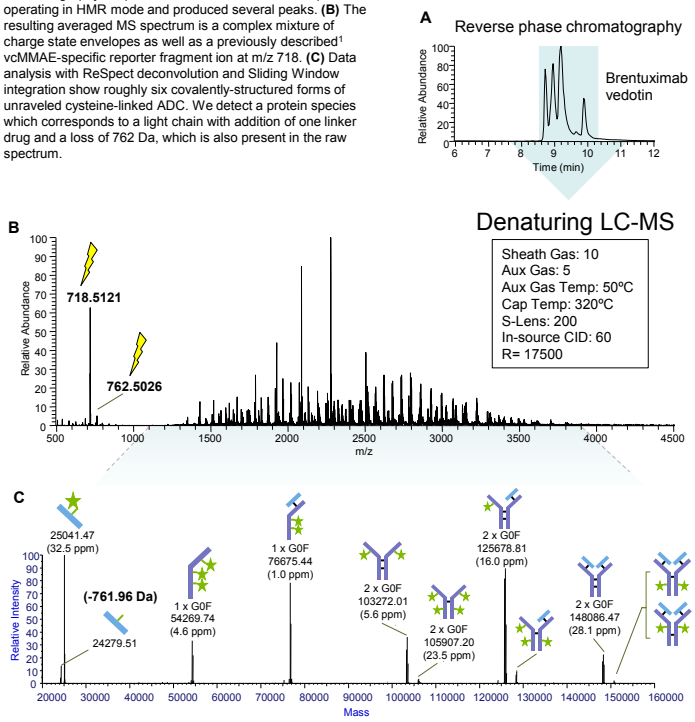
Figure 2. LC-MS Instrumentation for Complete ADC Characterization
All experiments were performed using a Vanquish UHPLC connected to a Exactive HF or Q Exactive Plus with High Mass Range (HMR) mode.

RESULTS

DENATURING LC-MS, CYSTEINE-LINKED ADC

Intact protein LC/MS analysis conventionally involves using mobile phases which are comprised of organic and acidic/basic pH, often suited specifically for reverse phase chromatography. This strategy can be useful for achieving high resolution protein separations. Conditions such as these, however, are not compatible for performing intact analysis on certain classes of compounds which require preservation of non-covalent bonds to maintain structural integrity, such as cysteine-linked ADCs. We demonstrate this phenomenon using the cysteine-linked ADC *Brentuximab vendotin*. Denaturing (reverse phase) LC/MS analysis of *Brentuximab vedotin* results in detection of roughly six unraveled forms **(Figure 3A-C)**. We observed a previously-reported[1] collisionally-induced m/z 718 fragment of the fragile vcMMAE linker-drug **(Figure 3B)**. Upon deconvolution we also observed a mass corresponding to light chain with addition of one vcMMAE and a loss of approximately 762 Da.

Figure 3. Denaturing LC-MS analysis
(A) Unmodified sample (1 ug) was analyzed by reverse phase chromatography coupled to a Q Exactive Plus Orbitrap MS operating in HMR mode and produced several peaks. **(B)** The resulting averaged MS spectrum is a complex mixture of charge state envelopes as well as a previously described[1] vcMMAE-specific reporter fragment ion at m/z 718. **(C)** Data analysis with ReSpect deconvolution and Sliding Window integration show roughly six covalently-structured forms of unraveled cysteine-linked ADC. We detect a protein species which corresponds to a light chain with addition of one linker drug and a loss of 762 Da, which is also present in the raw spectrum.



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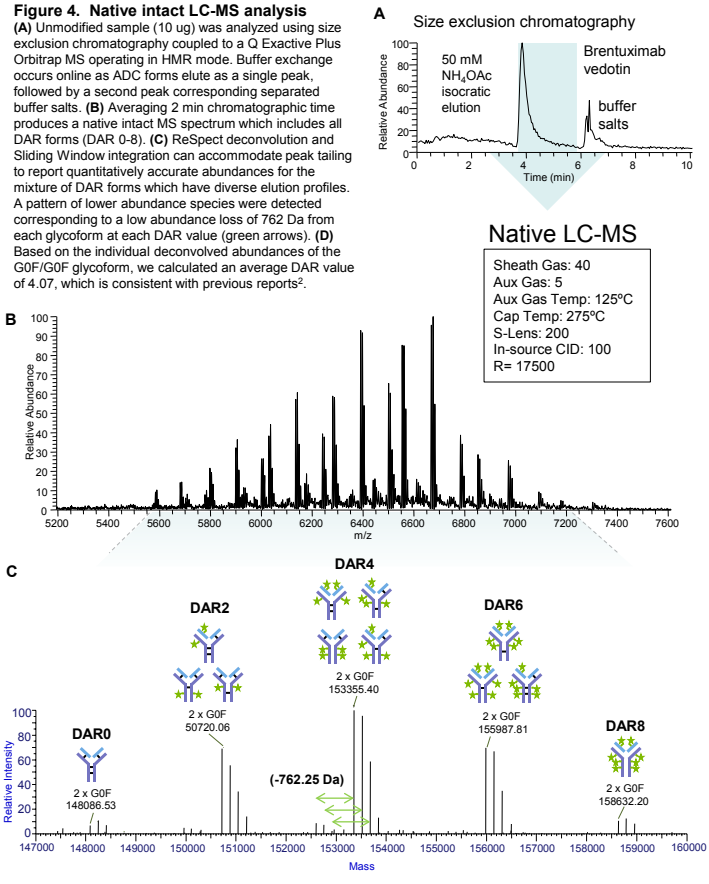
NATIVE INTACT LC-MS, CYSTEINE-LINKED ADC

Native MS intact protein analysis allows direct observation of molecules which rely on non-covalent interactions to preserve critical structural features, such as maintaining interchain associations which hold together cysteine-linked ADCs. The use of 100% aqueous physiological pH buffers in native MS analysis produces decreased charge states (increased m/z) and improves mass separation of heterogeneous mixtures. We performed native size exclusion LC-MS and observed 5 distinct species corresponding to intact *Brentuximab vedotin* with 0, 2, 4, 6, or 8 vcMMAE linker-drugs **(Figure 4)**. We measured an average drug-to-antibody ratio of 4.07, which is consistent with a previously published studies reporting 3.9-4.2 drugs per antibody[2] .

Figure 4. Native intact LC-MS analysis
(A) Unmodified sample (10 ug) was analyzed using size exclusion chromatography coupled to a Q Exactive Plus Orbitrap MS operating in HMR mode. Buffer exchange occurs online as ADC forms elute as a single peak, followed by a second peak corresponding separated buffer salts. **(B)** Averaging 2 min chromatographic time produces a native intact MS spectrum which includes all DAR forms (DAR 0-8). **(C)** ReSpect deconvolution and Sliding Window integration can accommodate peak tailing to report quantitatively accurate abundances for the mixture of DAR forms which have diverse elution profiles. A pattern of lower abundance species were detected corresponding to a low abundance loss of 762 Da from each glycoform at each DAR value (green arrows). **(D)** Based on the individual deconvolved abundances of the G0F/G0F glycoform, we calculated an average DAR value of 4.07, which is consistent with previous reports[2].



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G0F/G0F DAR	Mass Accuracy (ppm)	Relative Abundance
DAR0	11.7	6.77
DAR2	23.1	69.23
DAR4	22.4	100.00
DAR6	40.5	69.75
DAR8	17.6	10.61

Average Drug-to-Antibody Ratio (DAR)
4.07

PEPTIDE MAPPING, CYSTEINE-LINKED ADC

A fundamental component of biotherapeutic protein characterization is peptide mapping. Whereas intact mass analysis aims to detect the abundances and distributions of mass deviation combinations, peptide mapping allows highly sensitive analysis of site-specific sequence features. The vcMMAE linker-drug on *Brentuximab vedotin* poses particular challenges when attempting to identify drug conjugation sites. We prepared a sample for peptide mapping using reduction and alkylation to block non-drug-conjugated cysteines, followed by trypsin digestion. In one 90 min LC-MS gradient we were able to achieve 99% sequence coverage for both light and heavy chains and detect peptides spanning all four drug conjugation sites. HCD fragmentation allowed detection of a peptide in the hinge region of the heavy chain that is differentially modified with 0-2 vcMMAE drugs. As a result efficient elution requires sustained delivery of high organic mobile phase.

Figure 5. Peptide Mapping of Cysteine-Linked ADC *Brentuxmab vedotin*
Reduced, alkylated, and trypsin-digested sample (2.5 ug) was separated using a 90 min gradient and eluted into a Q Exactive HF (equipped with HMR mode) operating in Standard mode. Using a mass accuracy cut off of 5 ppm, we achieved 99% sequence coverage of both light and heavy chains. We detected known glycopeptides and were able to detect MMAE-conjugated peptides at all four cysteines (red circles) which are normally involved in interchain disulfide pairs in naked antibodies. A trypsin peptide sequence at the hinge region of the heavy chain (red asterisk) was present in forms ranging from 0-2 vcMMAE conjugations. A missed cleavage peptide contained up to 3 conjugations.

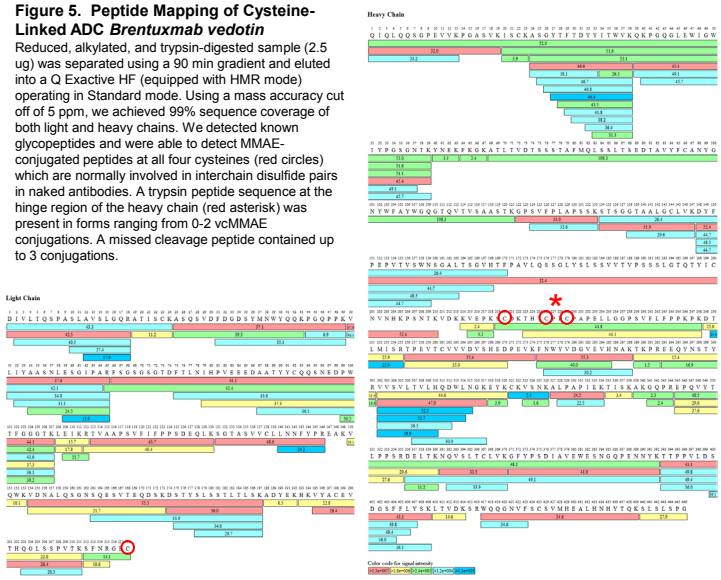


Figure 6. Hinge Region Peptide of Cysteine-linked ADC is Site of Multiple Conjugations
(A) Our data analysis in BioPharma Finder software resulted in detection of peptides which covered the hinge region of the heavy chain (red asterisk). A faithfully-trypsin-cleaved THT-KPK peptide was detected with 0-2 vcMMAE conjugations at cysteines (red circles) normally involved in interchain disulfide pairs. **(B)** Addition of vcMMAE to peptides dramatically increases hyphobicity which results in poor elution and increased retention time. MS/MS analysis of the **(C)** 1 linker-drug (both positional isomers) and **(D)** two linker-drug forms in BioPharma Finder allowed clear sequencing of y-ions in the hinge peptides, and thus facilitated automatic detection.

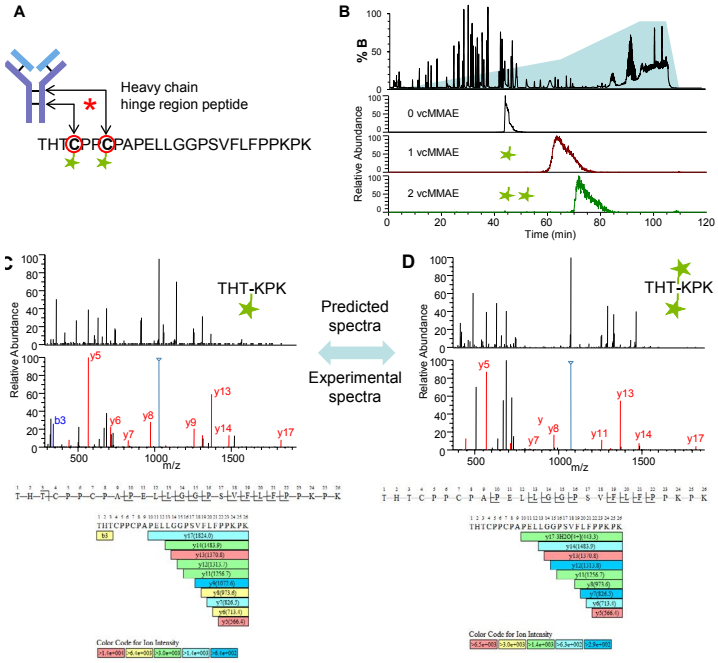
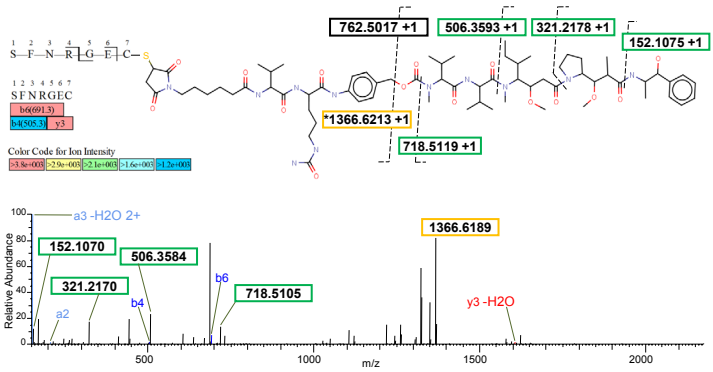


Figure 7. HCD Signature Fragment Ions for vcMMAE Linker-Drug
The light chain C-terminal peptide SFN-GEC is a conjugation site for vcMMAE. This modified peptide was automatically identified by BioPharma Finder (left side top panel). Further manual inspection produced additional fragment assignments for vcMMAE signature ions (right side top panel). Theoretical masses (top panel) were calculated manually and matched to experimental masses (bottom panel) within 5ppm (green boxes). A cleavage site for the loss of 762 Da is shown (black box; theoretical monoisotopic mass = 762.5017). We observed a high abundance ion at m/z 1366.6189 (orange box, asterisk) which corresponds to the peptide-retaining fragment pair of a 762 Da loss with an additional loss of 2 protons, presumably due to formation of a seven-membered aromatic ring.



CONCLUSIONS

•We have modified the control software in Q Exactive Plus and Q Exactive HF mass spectrometers to add native MS capability.

•Native LC/MS intact analysis of Brentuximab vedotin resulted in detection of intact ADC forms, DAR0-8. ReSpect deconvolution and Sliding Window integration showed an average DAR of 4.07, consistent with previous studies.

•Acquisition of MS/MS spectra with HCD fragmentation on Q Exactive Plus and Q Exactive HF Orbitrap mass spectrometers followed by data analysis with BioPharma Finder resulted in 99% sequence coverage from a single 90 min gradient using 5 ppm mass tolerance.

•Addition of vcMMAE linker drug dramatically increases peptide hydrophobicity and retention time.

•Signature HCD fragment ions of linker-drug may allow additional means for identifying drug-conjugated peptides.

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