POSTER NOTE

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Integrated characterization of a lysine-linked antibody-drug conjugate by native intact mass analysis and peptide mapping performed on a hybrid quadrupole-Orbitrap mass spectrometer with high mass range

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ABSTRACT

Challenges of antibody drug conjugate (ADC) characterization include measuring an accurate measurement of drug-to-antibody ratio using intact mass analysis and determining sites of drug conjugation using peptide mapping. We present the analysis of a lysine-linked ADC, Trastuzumab Emtansine, in which both peptide-mapping MS/MS and high resolution native-MS intact protein workflows are performed for the first time on a single commercial Orbitrap™ mass spectrometer.

INTRODUCTION

Therapeutic proteins, such as antibody-drug conjugates (ADCs), often present great analytical challenges. Complementary MS approaches such as peptide mapping and intact mass analysis are needed for complete characterization. The use of 100% aqueous buffers at neutral pH in native MS analysis will reduce charge state value and, as a result, the distribution of ions will be presented at higher m/z. Using native MS to improve mass separation of heterogeneous mixtures is a powerful strategy when considering analysis of complex biotherapeutics such as random-lysine-linked ADCs. We have utilized a benchtop quadrupole-Orbitrap mass spectrometer to perform both peptide mapping and high resolution native MS intact mass analysis. In this study we demonstrate integrated characterization of a lysine-linked ADC, utilizing high mass range for native MS functionality and standard mode for peptide mapping.

MATERIALS AND METHODS

Trastuzumab Emtansine was prepared without pretreatment for trypsin peptide mapping or native intact analysis. For intact mass analysis ADC samples in formulation buffer were desalted online using size exclusion chromatography (SEC) or reversed phase (RP) chromatography coupled directly to the mass spectrometer. For mass spectrometry we utilized a commercially-available Thermo Scientific™ Q Exactive™ Plus mass spectrometer with High Mass Range (HMR) mode to allow improved high mass transmission and scanning up to *m/z* 8000 for native intact analysis. MS1 spectra were collected using 10 microscans and a resolution setting of 17,500, 35,000, or 70,000. Intact mass spectra were deconvoluted using the ReSpect™ algorithm in BioPharma Finder 2.0. Deconvolution spectra were annotated was using the amino acid sequence of Trastuzumab, with a total of 16 disulfide bonds, a fixed modification of G0F/G1F, and a variable modification of MCC-DM1 (avg mass = 957.53 Da). DAR value was calculated using a fixed modification of G0F/G1F and a variable modification of MCC-DM1.

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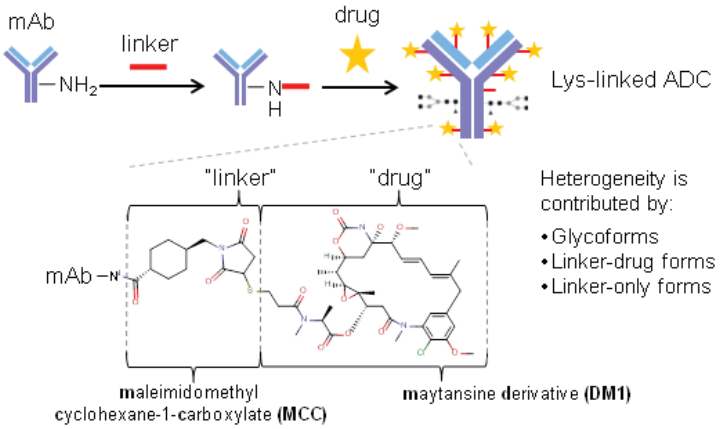
Samples were prepared for peptide mapping using SMART Digest™ followed by reduction using a final concentration of 20 mM DTT. Peptides mapping was performed in triplicate using 60 min RP gradients for separation, detected using a Q Exactive Plus mass spectrometer operated in standard mode at a resolution setting of 70,000. Peptide mapping data were searched using the MassAnalyzer algorithm in BioPharma Finder 2.0, using variable modifications of N-glycan (CHO-derived), deamidation (N, Q), oxidation (M, W), MCC-DM1 (K; mono mass = 956.3644), and "dead" MCC (K; mono mass = 221.1052). HCD fragmentation spectrum of KVEPK peptide was manually searched using a manual assignment within 5 ppm tolerance.

RESULTS

Native LC/MS analysis improves m/z separation of components in spectra of intact protein complex mixtures

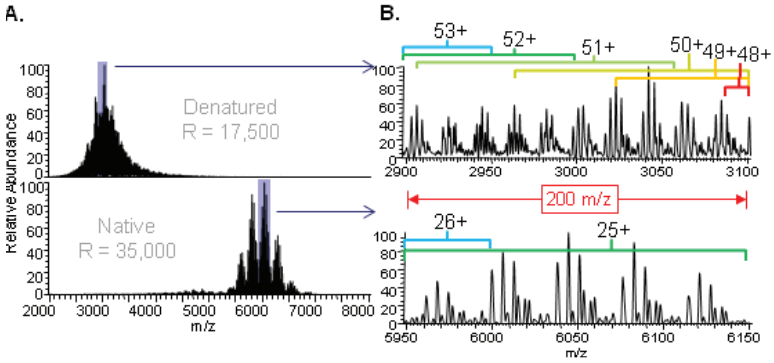
Antibody drug conjugates (ADCs) are complex mixtures. The diverse nature of ADC components is contributed by chemical modifications which arise from the manufacture of the core antibody as well as the drug conjugation chemistry (Figure 1). Major forms of lysine-linked ADCs differ in N-glycan composition, the number of linker-drugs attached, as well as potential linker-only attachments.

Figure 1 – Multi-stage assembly of lysine linked ADCs can result in a highly complex mixture.



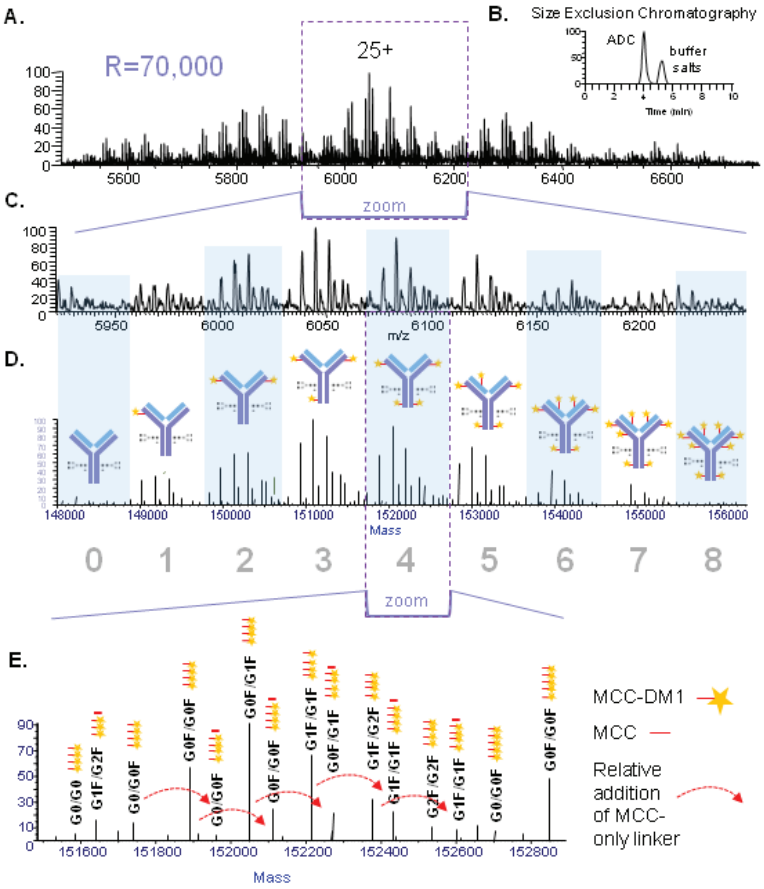
The lysine-linked ADC, Trastuzumab Emtansine, is observed in intact analysis as a complex mixture. Due to the broad diversity of components in ADC mixtures, denaturing MS approaches, such as reverse phase LC, result in extensive m/z interferences. Native MS allows greater m/z separation of sequential charge state envelopes, and is ideal for resolving complexity of co-eluting species (Figure 2).

Figure 2 – Native LC/MS analysis of intact proteins allows improved separation of mass peaks at higher m/z range. (A) Denaturing MS spectra (from reverse phase LC) are observed at lower m/z ranges while native MS spectra from online SEC are observed at higher m/z ranges. (B) A detailed view shows that 2-3 sequential charge state envelopes overlap compared to an overlap of 0-1 charge state envelopes in the native MS spectrum.



We measured the intact native average drug to antibody ratio (DAR) using online native SEC-MS. Intact analysis of the untreated ADC showed a distribution of 0-8 linker-drug conjugations with each DAR value showing an expected distribution of N-glycans (Figure 3). We additionally found a third layer of heterogeneity corresponding to a linker-only form of conjugation. Based on the most abundant glycoform we calculated an average DAR value of 3.71 (Figure 4). This value is consistent with, but slightly higher than, the previously observed value of 3.5 using static nanospray and native intact analysis **(Ref 1)**.

Figure 3 – Native size exclusion chromatography (SEC) coupled to mass spectrometry of "true" intact Trastuzumab Emtansine using High Mass Range mode on Q Exactive Plus with BioPharma Option. (A) Native intact Orbitrap MS spectra of Trastuzumab Emtansine acquired at R=70,000 setting. (B) Online SEC/MS allows automatic desalting of buffer salts which interfere with electrospray ionization. (C) A detailed view of the 25+ charge state accurately resembles (D) the ReSpect deconvolution results, showing a distribution of 0-8 MCC-DM1 linker-drug attachments. (E) A detailed view of the D4 cluster shows the presence of a MCC linker-only addition.



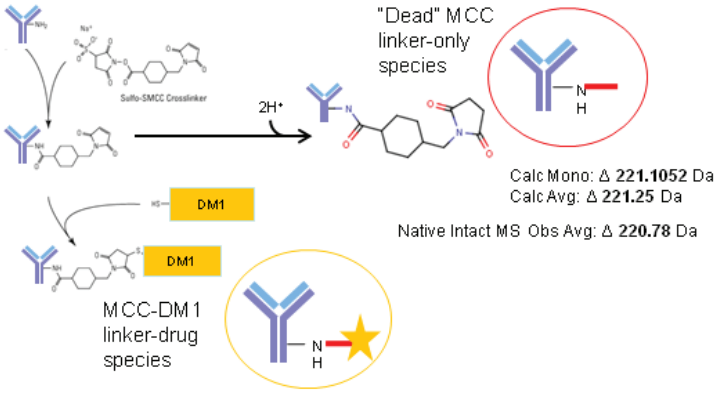
Average Drug-to-Antibody Ratio (DAR)	G0F/G1F DAR	Mass Accuracy (ppm)	Relative Abundance (%)
3.71	DAR0	6.49	9.19
	DAR1	21.69	34.26
	DAR2	0.05	59.03
	DAR3	6.81	100.00
	DAR4	5.17	91.16
	DAR5	6.69	67.42
	DAR6	15.20	40.46
	DAR7	6.28	24.28
	DAR8	3.78	3.84

Figure 4 – DAR calculation of Trastuzumab Emtansine based on most abundant glycoform (G0F/G1F).

Peptide mapping of Trastuzumab Emtansine allows localization of conjugation sites

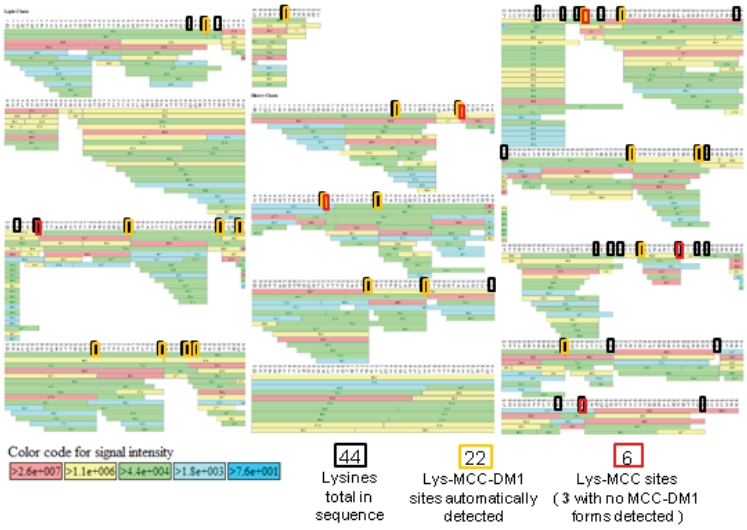
Native intact spectra of Trastuzumab Emtansine revealed a distribution of forms corresponding to combinations of distinct glycoforms, 0-8 MCC-DM1 linker-drug additions, and MCC linker-only additions. We observed the MCC linker-only forms as a mass shift of approximately 220.78 Da. This measurement was consistent with and very close to a possible "dead" linker form, which would lack reactivity due to the loss of a double bond and would contribute an additional two protons to the mass shift (Figure 5).

Figure 5 – A possible route of origin for a "dead" linker-only MCC species which was observed as an independent layer of heterogeneity in intact native MS spectra.



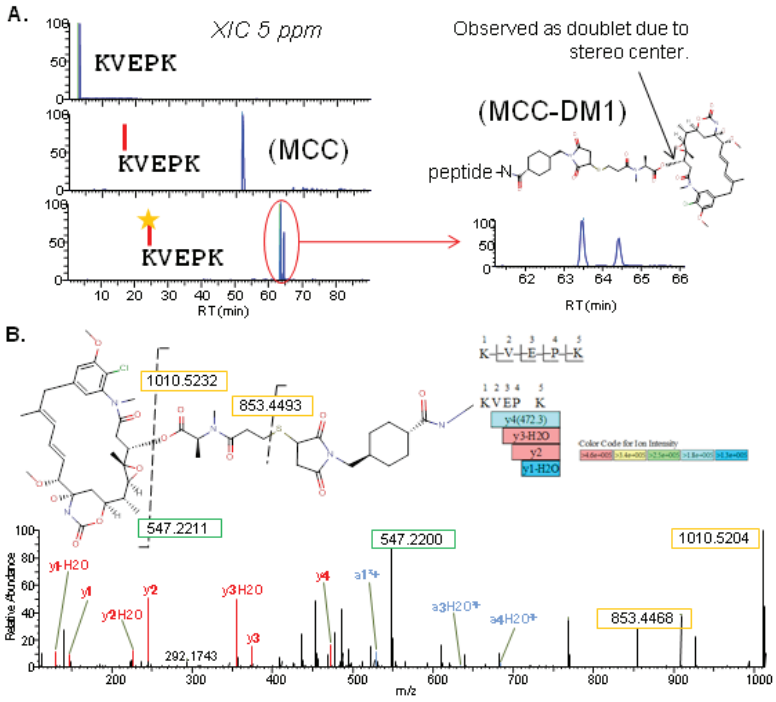
Trypsin peptide mapping analysis resulted in 100% sequence coverage (Figure 6). We identified 22 out of 44 lysine sites of MCC-DM1 linker-drug conjugation. Additionally, we observed several lysines with MCC linker-only conjugations, 3 of which we did not also detect the presence of any linker-drug forms. We observed that linker-only peptides eluted much later than unmodified peptides (Figure 7). MCC-DM1 linker-drug caused a further increase in retention time as well as elution as a doublet, due to the stereo center of DM1.

Figure 6 – Triplicate peptide mapping results showed many lysine conjugation sites for MCC-DM1 linker-drug additions as well as MCC linker-only additions.



HCD spectra show fragmentation of both the peptide as well as the linker drug. Signature fragment ions could be used to search for unexpected conjugated peptides **(Ref 2)**.

Figure 7 – Drug conjugation changes peptide behavior. (A) KVEPK peptide is unmodified, or modified with MCC linker-only or MCC-DM1 linker-drug. (B) HCD fragmentation produces signature ions specific to conjugated peptides.



CONCLUSIONS

- Native MS improves m/z separation of complex ADC spectra.
- Lysine-linked ADC mixtures may also include linker-only forms.
- Drug conjugation changes peptide behavior in peptide mapping
- HCD fragmentation generates conjugate-specific signature ions.

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