Comprehensive Characterization of Site-specific Engineered Antibody Drug Conjugate by Orbitrap Mass Spectrometer

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Overview

Purpose: Confidently characterize drug payloads to antibody heavy chain glycans by Orbitrap technology.

Methods: ADCs were made from Thermo Scientific™ SiteClick™ enzyme-based N-glycan labeling of trastuzumab with DIBO-MMAE toxin. Intact, F(ab')$_2$, scFc fragments and tryptic peptides of wild type antibody, azide-tagged intermediate, and ADCs were analyzed on Thermo Scientific™ Q Exactive™ Plus MS and Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometers by LC-MS. Intact and fragment data were processed by Thermo Scientific™ Protein Deconvolution™ 4.0 software. Peptide data was processed by Thermo Scientific™ PepFinder™ 2.0 software.

Results: MMAE-modified trastuzumab sample was separated by liquid chromatography on a Thermo Scientific™ MabPac™ RP column. The conjugation is a mixture of 0, 1, 2, 3, and 4 MMAE per antibody. The average DAR is calculated as 3.2. The deconvoluted monoisotopic mass of Fc/2 demonstrated accurate assignment of two attached MMAE toxin drugs and linkers to the antibody, while the mass of the F(ab')$_2$ portion for both azide and MMAE-labeled samples remain the same. The drug conjugation site was confirmed by peptide mapping, but the drug and linker stability should also be considered in the peptide map analysis due to potential modification or cleavage during sample preparation.

Introduction

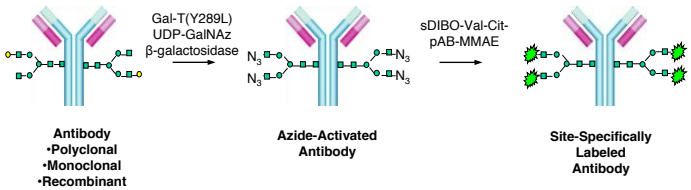
Antibody drug conjugates (ADCs) are becoming an increasingly common approach for drug therapy. To date, three ADC drugs have been approved by the U.S. FDA. Another 10 ADCs in the pipeline are in late-stage clinical development. From a safety and efficacy perspective, ensuring the integrity of antibody-cytotoxin conjugate during drug development and production is critical. Among the several types of conjugation chemistries, enzyme-based site-specific modification shows great potential by eliminating the interruption of the antibody-antigen interaction and provides a highly reproducible and modular conjugation system when compared to standard lysine and cysteine conjugation. Here we report the characterization of ADCs with enzymatic labeled antibody N-glycans using the Q Exactive Plus MS and Orbitrap Fusion mass spectrometers.

Methods

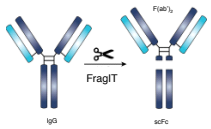
Sample Preparation

ADCs were made from SiteClick enzyme-based N-glycan labeling of trastuzumab with DIBO-MMAE toxin(Scheme 1). The trastuzumab antibody was incubated with β-galactosidase, Gal-T(Y289L), and UDP-GalNAz . Excess UDP-GalNAz was removed by molecular weight cut-off spin filters or dialysis prior to click conjugation reactions. Then a dibenzocyclooctyne (DIBO)-MMAE toxin was added to the azide-activated antibodies. The resulting conjugates were dialyzed in 50mM ammonium acetate for MS analysis. One portion were cleaved by FabRICATOR™ (FragIT™) enzyme to generate F(ab')$_2$ and scFc fragments (Scheme 2). The other portion was denatured, reduced, alkylated and enzymatic digested by trypsin.

SCHEME 1. SiteClick Enzyme-based N-glycan Labeling of Antibody



SCHEME 2. Cleavage of mAb to form F(ab')$_2$ and scFc Fragments



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Liquid Chromatography

Intact and fragment samples were separated and analyzed on a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system coupled to the Q Exactive Plus MS by LC-MS using a MabPac-RP (3x50mm,4μm, heated at 80°C, PN: 088645) column.

Azide activated intermediate and MMAE conjugated glycopeptide analysis was conducted by LC-MS on a Orbitrap Fusion system using a Thermo Scientific™ Acclaim™ RSLC(2.1x 100mm, 2.2 µm, heated at 60°C) column.

For ADC fragment separation, the gradient elution was performed from 10–30% over 1 min, from 30–55% over 5 min and from 55-95% over 1 min with ACN in 0.1% formic acid at a flow rate of 400 uL/min.

For glycopeptide analysis, the gradient elution was kept at 0.1% over 5 min, from 0.1–35% over 85 min and from 35-95% over 5 min with ACN in 0.1% formic acid at a flow rate of 300 uL/min.

Mass Spectrometry

The Q Exactive Plus MS interfaced with H-ESI II ion source was employed for intact and fragment MS analysis. The acquisition method was set with a full scan at both 17,500 (FWHM, at *m/z* 200) and 280,000 resolution in positive mode. The method parameters were: AGC 3e6, IT 200 ms, in-source CID 55ev, scan range: 1000-4000 *m/z*, spray voltage 3.8kv, sheath gas 50, aux gas 15, capillary temperature 320°C, s-lens 50, probe heater temperature 300°C.

An HCDpdETD method was employed on the Orbitrap Fusion mass spectrometer with H-ESI source for glycopeptide analysis to identify the conjugation site at the peptide level. Full scan data was acquired at a resolution of 120,000 , mass range 400-1600 *m/z*, AGC 4e5, and IT 50 ms. Data dependent Top10 MS/MS (HCD, NCE 30%) for peptide with charges 3-8 were acquired at resolution of 30,000, AGC 5e4, and IT 60 ms. ETD of the same peptide precursor was triggered if any of glycan oxonium ions(m/z 204. 0867-HexNac, 138.0545-HexNac fragment and 366.1396-HexNacHex) was detected in HCD MS/MS spectrum. EThcD was performed using calibrated reaction times at resolution of 30,000 with supplementary activation energy of 15%, AGC 1e5, IT 250 ms scan range 120-2000 *m/z*. Other parameters include quadrupole isolation of 2 m/z, s-lens 60, spray voltage 3600V, sheath gas 50, aux gas 20, ion transfer tube 325°C, vaporizer temp 100°C.

Data Analysis

Average Intact mass of wild type antibody, azide-tagged intermediate, and ADCs as well as F(ab')$_2$ fragment were analyzed by Protein Deconvolution 4.0 software using the ReSpect algorithm. Monoisotopic mass of scFc was determined by the Xtract algorithm. Glycopeptides with/out drug/azide were identified by PepFinder 2.0 software.

Results

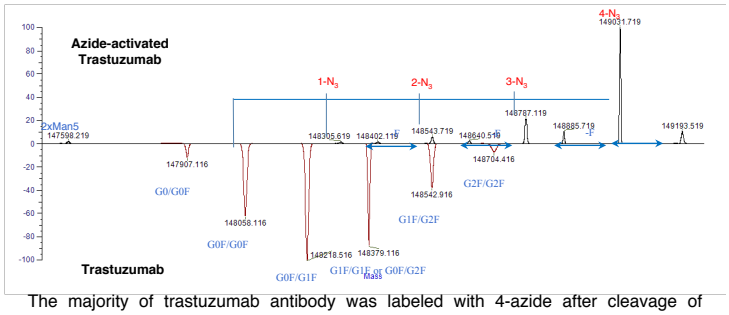
Intact Mass Analysis

Trastuzumab was used as the model antibody to synthesize ADC with DIBO-MMAE. Wild type, azide-activated, and MMAE-labeled antibodies and their corresponding F(ab')$_2$ and scFc fragments were analyzed by HRAM full scan analysis. Deconvoluted spectra of intact wild antibodies as expected show major glycoforms of trastuzumab with highly accurate mass measurements (Table 1).

TABLE 1. Mass Measurement Accuracy of Deconvoluted Glycoforms of Trastuzumab

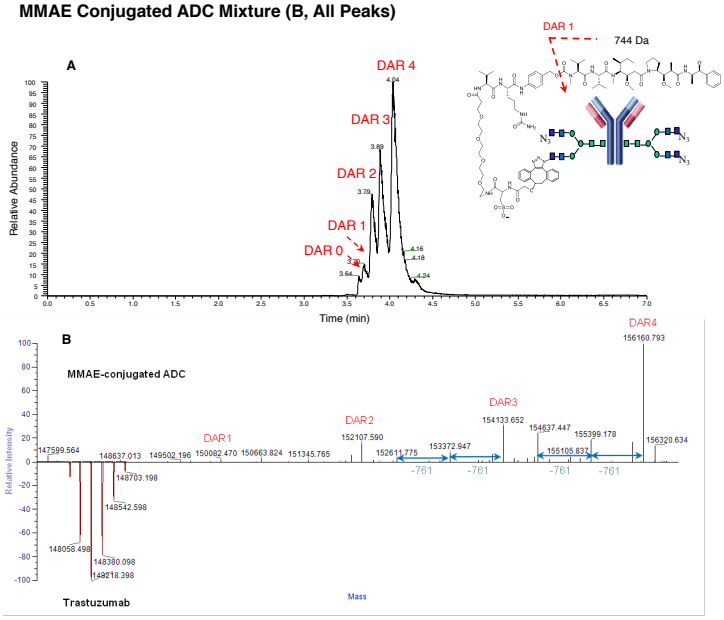
	G0/G0F	G0F/G0F	G0F/G1F	G1F/G1F or G0F/G2F	G1F/G2F	G2F/G2F
Theoretical Mass	147910.4	148056.6	148218.7	148380.8	148543.0	148705.1
Measured Mass	147907.1	148058.1	148218.5	148379.1	148542.9	148704.4
Mass Error (ppm)	22	10	1.3	11	0.7	4.7

FIGURE 1. Intact Mass Analysis of Azide-activated and Unlabeled Trastuzumab



The majority of trastuzumab antibody was labeled with 4-azide after cleavage of terminal galactose with β-(1-4) galactosidase and labeling with GalT(Y289L) (Figure 1).The azide-labeled glycans are less heterogeneous than the unmodified ones and differ only by fucose (-F) and terminal azide (-N_3). The ADC sample with DIBO-MMAE conjugation to the azide activated trastuzumab was separated on MacPac-RP column (Figure 2A). Sliding window deconvoluted mass spectrum of all peaks in Figure 2A shows MMAE drugs were conjugated to antibody at different ratios (Figure 2B) with the majority of 4 drugs per antibody as expected (Figure 2B and 2C). Average DAR was calculated as 3.2 based on baseline separation of different form of conjugations under optimized LC condition (data not shown). Due to the applied in-source CID 55ev, DIBO-Val-Cit-pAB-MMAE was fragmented at the indicated ester bond (Scheme 2A insert, red dash arrow). The loss of 761 Da is the combination of 744 Da and subsequent water loss.

FIGURE 2. Chromatographic Separation (A) and Deconvoluted Mass Spectra of MMAE Conjugated ADC Mixture (B, All Peaks)



The FabRICATOR enzyme fragments from the above samples were well separated by the optimized LC conditions (Figure 3). The deconvoluted monoisotopic mass comparison data (Figure 4) further demonstrated the successful conjugation of 2 MMAE on the antibody heavy chain (scFc), while the F(ab')$_2$ stays the same. The glycan in-source fragments, loss of GalNAz, GlcNAc and Manose were observed in mass spectra, which can be minimized in future study by decreasing or not using in-source CID.

FIGURE 3. Chromatographic Separation of (Fab')$_2$ and scFc Fragments in Unlabeled(A), Azide-activated(B) and MMAE labeled Trastuzumab(C)

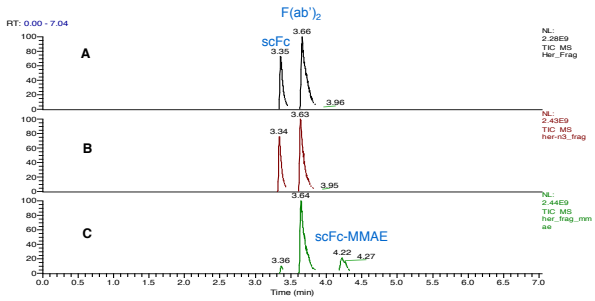
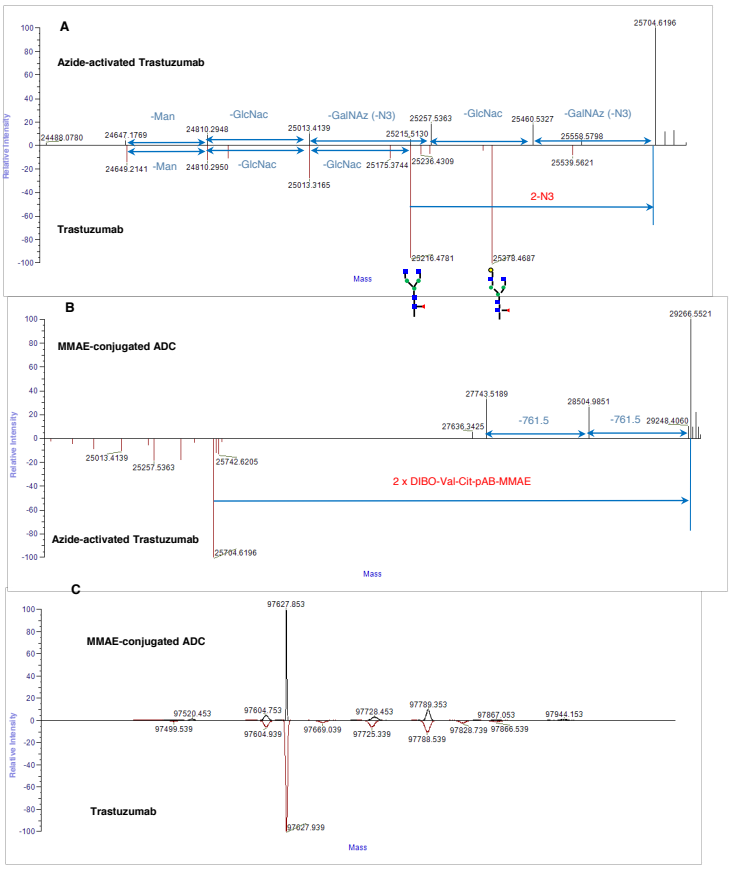


FIGURE 4. Monoisotopic Mass Comparison of scFc between Azide-activated and Unlabeled (A), MMAE-conjugated and Azide-activated Trastuzumab (B) and Average Mass Comparison of F(ab')$_2$ between MMAE-conjugated and Unlabled Trastuzumab (C)

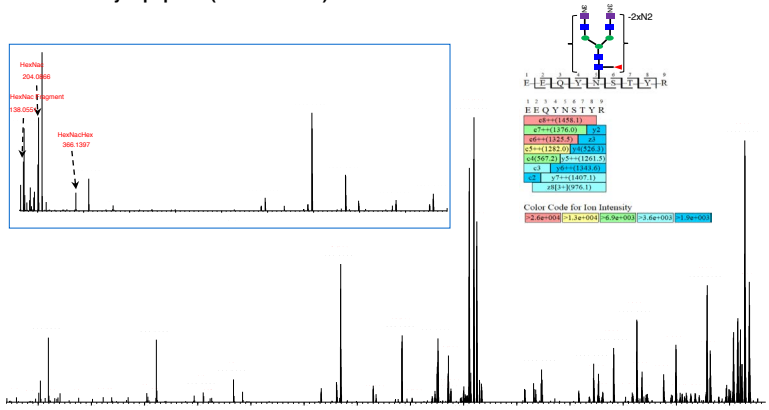


Peptide Mapping-ADC Conjugation Site Analysis

To confirm the azide and MMAE conjugation site of the antibody, reduced and alkylated, trypsin digested samples were analyzed using LC-MS/MS on the Orbitrap Fusion mass spectrometer by HCDpdETD. For the azide-activated intermediate, the addition of azide derivative to Asn 300 residue is supposed to increase the mass of the corresponding peptide by 1934.7097 Da. However, the detected mass increase of this peptide was only 1906.7036 or 1880.7130 Da. The azide intermediate was converted to an amine during DTT reduction resulting the loss of 1 or 2 N2 (up to 56.0062 Da)[1].The annotated ETD spectrum from PepFinder clearly shows the addition of 2 x GalNAz (2x-N_3) to Asn 300 residue of G0F containing peptide EEQYNSTYR (Figure 5). The insert HCD spectrum of the same peptide shows presence of oxonium ions (in red color) cleaved from glycans and few peptide backbone fragmentation ions.

For ADC samples, the desired MMAE conjugation on peptide was not found. It may result from (1) The structure of drug payload and linkers might be easily fragmented during ionization based on the observance of fragment ions(loss of 761 m/z) under in-source CID. (2) Val-Cit di-peptide linker might be undergo protease cleavage.

FIGURE 5. ETD and HCD (Insert) Mass Spectra of *m/z* 768.8116, [M+4H]$^{4+}$ of Azide Modified Glycopeptide (EEQYNSTYR) with the Loss of 2x N2



Conclusion

The drug payload to antibody heavy chain N-glycans have been characterized by high resolution/accurate mass Orbitrap mass spectrometry on intact, fragment and peptide levels.

- Intact results show the majority of trastuzumab antibody exists in the 4-azide activation state after cleavage of terminal galactose with β-(1-4) galactosidase. The average DAR of MMAE-modified trastuzumab sample was calculated as 3.2.
- The deconvoluted monoisotopic mass of scFc demonstrated the accurate conjugation of 2 MMAE drug and linkers per antibody heavy chain, while the average mass of F(ab')$_2$ part of both azide and MMAE-labeled samples stays the same.
- Characterization of conjugation site at peptide level is complicated due to the modifications introduced during reduction, alkylation and trypsin digestion steps.
- Overall, intact and scFc, F(ab')$_2$ fragments mass analysis will be major approach for ADCs characterization. Top/Middle down provide complementary approaches. The structure and stability of drug payload and linkers should be considered for peptide level experiments.

Reference

1. Handlon A.L. and Oppenheimer N.J. Pharmaceutical Research, vol 5, No 5, 1988, 297-299.

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