Orbitrap Based Mass Spectrometric Characterization of Antibody Drug Conjugates Engineered through Antibody Glycans

Hongxia Jessica Wang,[1] Terry Zhang,[1] Brian J. Agnew,[2] Rosa Viner,[1] Shanhua Lin,[3]
Stephane Houel,[1] Jonathan Josephs[1]
[1]Thermo Fisher Scientific, San Jose, USA; [2]Thermo Fisher Scientific, Eugene, OR;
[3]Thermo Fisher Scientific, Sunnyvale, CA

Poster Note 64397

Overview

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

Methods: ADCs were made from SiteClick™ enzyme-based N-glycan labeling of tratuzumab 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 and Thermo Scientific™ Orbitrap Fusion™ 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. Top down sequencing was performed by Prosight Lite software from Northeastern University.

Results: MMAE-modified trastuzumab sample was separated by liquid chromatography on 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. Top down analysis of intact single chain Fc (scFc) of mAb, azide activated intermediate and ADCs clearly shows the conjugation of linker and MMAE on antibody N-glycans. The drug conjugation site was also confirmed by peptide mapping, however the drug and linker stability should also be considered in the peptide map analysis due to potential modification or cleavage during sample preparation.

Introduction

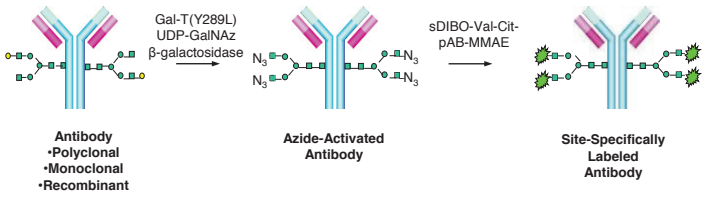
Antibody drug conjugates (ADCs) are becoming an increasingly common approach for drug therapy. To date, three ADC drugs have been approved by the U.S. FDA. Another 10 ADCs in the pipeline are in late-stage clinical development. From a safety and efficacy perspective, ensuring the integrity of antibody-cytotoxin 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 Thermo Scientific™ Q Exactive™ Plus and Orbitrap Fusion™ mass spectrometers.

Methods

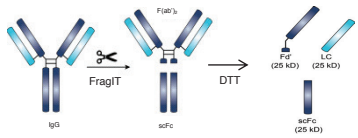
Sample Preparation

ADCs were made from SiteClick™ enzyme-based N-glycan labeling of Trastuzumab with DIBO-MMAE toxin(Scheme 1). The Herceptin 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 of 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. Generation of $F(ab')_2$, scFc, Fd' and LC Fragments



Liquid Chromatography

Intact and fragment samples were separated and analyzed on Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system coupled to the Thermo Scientific Q Exactive Plus instrument by LC- MS using a Thermo Scientific MabPac-RP (2.1x50mm,4μm, heated at 80°C, PN: 088648) 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.

Thermo SCIENTIFIC

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 0ev, 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 200°C.

An HCDpdETD method was employed on Orbitrap Fusion™ Tribrid™ 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 and 20%, 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. Middle down experiment was performed by targeted MS/MS at resolution 12,000, isolation window 3 *m/z*, AGC 5e5, IT 250ms, 5 microscans.

Data Analysis

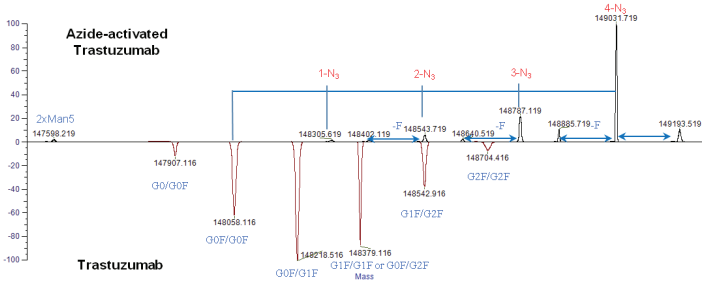
Average Intact mass of wild type antibody, azide-tagged intermediate, and ADCs as well as $F(ab')_2$ fragment were analyzed by Protein Deconvolution 4.0 using the Respect algorithm. Monoisotopic mass of scFc was determined by Xtract algorithm. Glycopeptides with/out drug/azide were identified by PepFinder 2.0 software. Top down sequencing of scFc and confirmation of DIBO-MMAE at antibody N-glycan were performed by Prosight Lite.

Results

Intact Mass Analysis

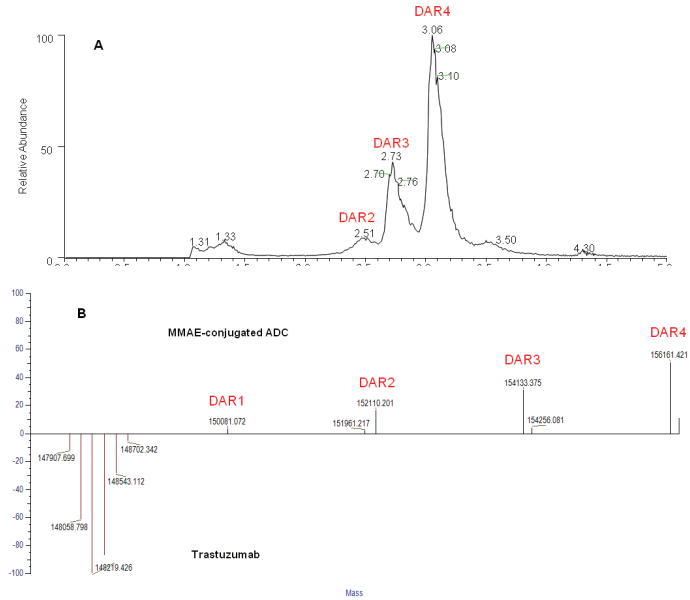
Trastuzumab was used as the model antibody to synthesize ADC with DIBO-MMAE. Wild type, azide-activated, and MMAE-labeled antibodies and their corresponding $F(ab')_2$ and scFc fragments were analyzed by HRAM full scan analysis. Deconvoluted spectra of intact wild antibodies show major glycoforms of Trastuzumab were all within 15ppm(Figure 1 bottom). 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 ($-N_3$).

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



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). Average DAR was calculated as 3.2 based on baseline separation of different form of conjugations under optimized LC condition.

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(data not shown). The glycan in-source fragments, loss of GalNAz, GlcNAc and Manose were observed in mass spectra when using in-source CID of 55ev, which can be eliminated in future study without use of in-source CID.

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

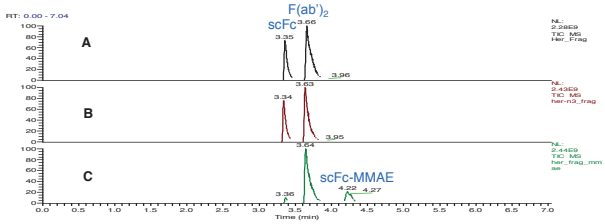
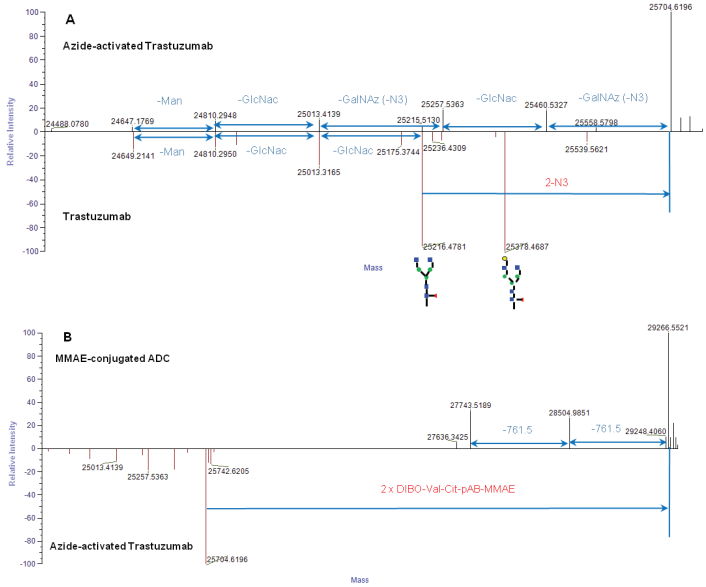


FIGURE 4. Monoisotopic Mass Comparison of scFc between Azide-activated and Unlabeled (A), MMAE-conjugated and Azide-activated Trastuzumab (B)



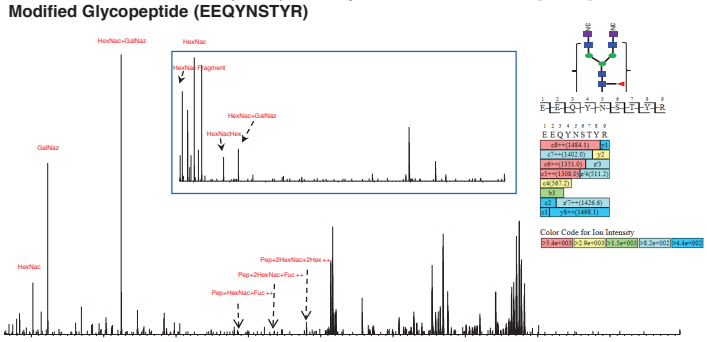
Peptide Mapping--ADC Conjugation Site Analysis

To confirm the azide and MMAE conjugation site of the antibody, reduced and alkylated, trypsin digested samples were first 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 1932.6951 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].

Based on above results, another set of non-reduced sample was prepared and analyzed by the same method. Azide modified glycopeptide was identified based on accurate mass and 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 under current conditions. It may result from (1) The enzymatic digestion efficiency of non-denatured and non-reduced ADC decreased significantly due to the conjugated DIBO-MMAE on the mAb. (2) Val-Cit di-peptide linker might be undergo protease cleavage. More investigations are needed to figure out the reason.

FIGURE 5. EThcD and HCD (Insert) Mass Spectra of *m/z* 781.5598, $[M+4H]^{4+}$ of Azide Modified Glycopeptide (EEQYNSTYR)

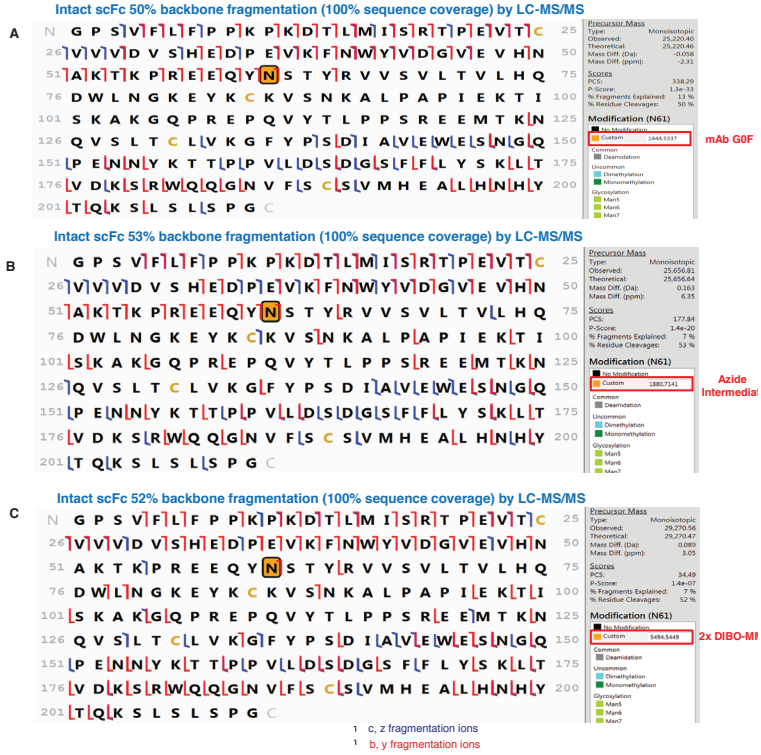


Top Down Sequencing--ADC Conjugation Site Analysis from Intact Large Fragment

To confirm that DIBO-MMAE was conjugated at mAb N-glycan, top down experiment was performed for Fabricator treated and further reduced wide type mAb, azide activated intermediate and ADCs samples. LC, scFc and Fd' fragments are well separated on Macpac-RP column and full mass spectrum of each fragment was acquired at 120k resolution. One or two multiple charged precursor ions were fragmented with HCD and EThcD methods. The backbone fragmentation of scFc of unconjugated mAb, azide activated and MMAE conjugated ADCs are 50, 53 and 52%, respectively with 10ppm mass tolerance window. The full sequence coverage of all samples is achieved (Figure 6). The loss of 2x N2 for azide activated Trastuzumab at Asn 300 was observed due to the reduction of disulfide bonds of $F(ab')_2$ to form Fd' and LC (Figure 6B). Figure 6C clearly demonstrated the DIBO-MMAE was conjugated to Asn 300 of heavy chain N-glycans.

Fd' and LC fragments were also sequenced during the same LC-MS/MS run. The backbone fragmentation of both Fd' and LC is above 50% and 100% sequence coverage is achieved (data not shown).

FIGURE 6. Top Down Sequencing of scFc of Wild Type Trastuzumab (A), Azide Activated (B) and MMAE Conjugated (C) Trastuzumab by LC-MS/MS



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.
- The structure of linker and drugs need to be carefully considered when characterizing conjugation site at peptide level due to the possible modifications introduced during reduction, alkylation and trypsin digestion steps.
- Top down sequencing of ADC intact large fragment shows its great potential for conjugation site analysis with minimum sample preparation and far reduced sample analysis time especially when the linker is not stable during peptide sample preparation.

Reference

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

www.thermofisher.com

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Canada +1 800 530 8447
China 800 810 5118 (free call domestic) 400 650 5118
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 10 3292 200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591
Japan +81 45 453 9100
Korea +82 2 3420 8600
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00
Russia/CIS +43 1 333 50 34 0
Singapore +65 6289 1190
Spain +34 914 845 965
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
UK +44 1442 233555
USA +1 800 532 4752

PN64397-EN 111/16S

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand