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High Resolution LC/MS Separation and Characterization of Chemoenzymatic Site-specific Engineered Antibody-Drug Conjugates

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ABSTRACT

Purpose: Demonstrate application of the SiteClick™ technology to the production of antibody-drug conjugates (ADCs) and a number of different detection probes.

Methods: ADCs were made from SiteClick™ enzyme-based N-glycan labeling of bevacizumab and panitumumab with DIBO-MMAE and DIBO-DFO. F(ab')$_2$ and scFc fragments from unconjugated, azide-tagged intermediate, and ADCs were analyzed using a Thermo Scientific™ MAbPac™ RP column coupled with a Thermo Scientific™ Orbitrap™ Fusion™ mass spectrometer.

Results: Enzymatic pre-treatment, or "trimming" of the antibody glycans with GlycINATOR® endoglycosidase S2, prior to azide activation, results in highly-reproducible degrees of labeling (DOLs) of 2.0.

INTRODUCTION

Antibody drug conjugates (ADCs) are becoming a powerful addition to monoclonal antibody drug therapies. From a safety and efficacy perspective, ensuring the integrity of antibody-cytotoxin conjugate during drug development and production is important. ADCs created using standard lysine and cysteine conjugation methods have multiple Drug-to-Antibody (DAR) forms. Chromatographic separation of multiple DAR forms is challenging due to the sample heterogeneity. Among the several types of conjugation chemistries, enzyme-based site-specific modification shows great potential by eliminating the interruption of antibody-antigen interaction, and providing a highly reproducible and modular conjugation system when compared to standard lysine and cysteine conjugation.

MATERIALS AND METHODS

Enzyme Labeling

In a one-pot activation reaction, antibodies were incubated for 5 hours at 37º C with the GlycINATOR enzyme (Genovis) for 1 hour at 37º C, then UDP-GalNAz substrate, β-Gal-T1(Y289L) enzyme, and $MnCl_2$ were added, and the mixture was incubated overnight (16 hrs) in non-phosphate buffer (pH 7.4) at 30 ºC. Excess UDP-GalNAz and β-Gal-T1(Y289L) were removed using 50 kD MW cut-off Amicon™ Ultra spin filters (MilliporeSigma). The purified azide-activated antibodies were labeled with DIBO-MMAE, or DIBO-DFO at 20 ºC (Scheme I).

Purification of Labeled mAb and mAb fragments

Antibodies were cleaved using FragIT™ MicroSpin columns (Genovis) to generate scFc and F(ab')$_2$ fragments. All antibody samples, cleaved and non-cleaved, were exhaustively dialyzed into 50 mM ammonium acetate buffer (pH 6.0) and then dried in the speedvac for downstream LC/MS analyses.

LC/UV Analysis

Intact and fragment samples were separated and analyzed on Thermo Scientific™ Vanquish™ UHPLC system, using a Thermo Scientific™ MAbPac™ RP analytical column, 4.0 µm, 1.0 × 150 mm column (p/n 302598) and H_2O/TFA/acetonitrile mobile phase at 80 ºC.

LC/MS Analysis

Intact and fragment samples were separated and analyzed on the Vanquish UHPLC system coupled to a Thermo Scientific™ Orbitrap Fusion™ Lumos Tribrid MS instrument, using a Thermo Scientific™ MAbPac™ RP analytical column, 4.0 µm, 2.1 × 50 mm column (p/n 088648) and H_2O/FA/acetonitrile mobile phase at 80 ºC. The MS acquisition method was set with a full scan at both 15,000 (FWHM, at *m/z* 200) and 120,000 resolution in positive mode. The method parameters were: AGC 2e^5, IT 200 ms, in-source CID 0 ev and 35 ev, scan range: 800-3000, 1000-3500 *m/z*; spray voltage 3.8 kV, sheath gas 60, aux gas 20, capillary temperature 350 ºC, EDIF 30, probe heater temperature 150 ºC.

Data Analysis

Average masses of scFc and F(ab')$_2$ fragments from unmodified antibody, azide-tagged intermediate, and ADCs were analyzed by Thermo Scientific™ BioPharma Finder™ 2.0 software using the Xtract algorithm for isotopically resolved MS spectra and the ReSpect™ algorithm for isotopically unresolved MS spectra.

RESULTS

Bevacizumab (IgG1) and panitumumab (IgG2) were used as the model antibodies to synthesize ADCs with DIBO-MMAE and DIBO-DFO. There are 2 N-glycans per antibody Fc domain, one on each heavy chain. After "trimming" of the antibody glycans with the GlycINATOR enzyme, there is one labeling site available per heavy chain. Therefore, up to 2 MMAE (or DFO) molecules can be conjugated to 1 mAb molecule. HPLC analyses of bevacizumab (Figure 1) and panitumumab (Figure 2) scFc and F(ab')$_2$ fragments before and after GlycINATOR glycan cleavage and SiteClick labeling shows complete conversion of labeled scFc domains.

HPLC profile of the scFc fragment showed that there are "twin peaks" in each case, regardless of the mAb (bevacizumab or panitumamab) or conjugated molecules (MMAE or DFO). High resolution LC/MS analysis demonstrated that these "twin peaks" have identical masses (Figure 3 for bevacizumab and Figure 4 for panitumab), indicating that they are structural isomers. NMR studies have shown that DIBO enantiomers were formed after the conjugation.

MS spectra of scFc fragments were acquired at 120K resolution and therefore the deconvolution were carried out using Xtract algorithm which is designed for isotopically resolved peaks. The deconvoluted spectra of scFc and its derivatives (treated with GlycINATOR, labeled with azide, and conjugated to MMAE or DFO) are shown in Figure 5 (bevacizaumab) and Figure 6 (panitumumab). MS spectra of F(ab')$_2$ fragments were acquired using 15K resolution and therefore the deconvolution was carried out using ReSpect algorithm which is designed for isotopically unresolved peaks. The deconvoluted spectra of F(ab')$_2$ are shown in Figure 7. After GlycINATOR cleavage and SiteClick labeling, bevacizumab and panitumumab F(ab')$_2$ fragments remain completely unaltered ensuring preservation of antigen binding.

SCHEME 1. SiteClick™ site-specific labeling of antibodies, conjugation to the chitobiose core

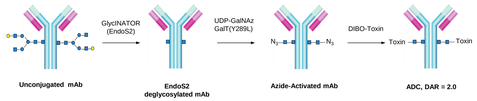
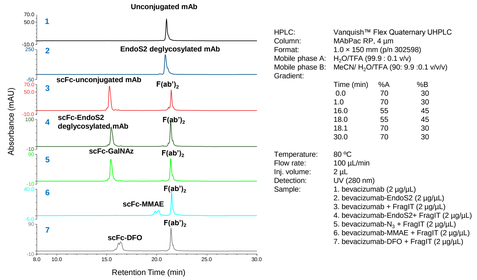


Figure 1. HPLC analyses of bevacizumab scFc and F(ab')$_2$ fragments before and after GlycINATOR glycan cleavage and SiteClick labeling

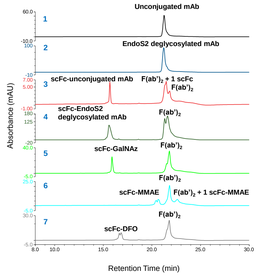


HPLC: Vanquish™ Flex Quaternary UHPLC
Column: MAbPac RP, 4 µm
Format: 1.0 × 150 mm (p/n 302598)
Mobile phase A: H_2O/TFA (99.9 : 0.1 v/v)
Mobile phase B: MeCN/ H_2O/TFA (90: 9.9 :0.1 v/v/v)
Gradient:

Time (min)	%A	%B
0.0	70	30
1.0	70	30
16.0	55	45
18.0	55	45
18.1	70	30
30.0	70	30

Temperature: 80 ºC
Flow rate: 100 µL/min
Inj. volume: 2 µL
Detection: UV (280 nm)
Sample:
1. bevacizumab (2 µg/µL)
2. bevacizumab-EndoS2 (2 µg/µL)
3. bevacizumab + FragIT (2 µg/µL)
4. bevacizumab-EndoS2+ FragIT (2 µg/µL)
5. bevacizumab-N_3 + FragIT (2 µg/µL)
6. bevacizumab-MMAE + FragIT (2 µg/µL)
7. bevacizumab-DFO + FragIT (2 µg/µL)

Figure 2. HPLC analyses of panitumumab scFc and F(ab')$_2$ fragments before and after GlycINATOR glycan cleavage and SiteClick labeling.



HPLC: Vanquish™ Flex Quaternary UHPLC
Column: MAbPac RP, 4 µm
Format: 1.0 × 150 mm (p/n 302598)
Mobile phase A: H_2O/TFA (99.9 : 0.1 v/v)
Mobile phase B: MeCN/ H_2O/TFA (90: 9.9 :0.1 v/v/v)
Gradient:

Time (min)	%A	%B
0.0	70	30
1.0	70	30
16.0	55	45
18.0	55	45
18.1	70	30
30.0	70	30

Temperature: 80 ºC
Flow rate: 100 µL/min
Inj. volume: 2 µL
Detection: UV (280 nm)
Sample:
1. panitumumab (2 µg/µL)
2. panitumumab-EndoS2 (2 µg/µL)
3. panitumumab + FragIT (2 µg/µL)
4. panitumumab-EndoS2 + FragIT (2 µg/µL)
5. panitumumab-N_3 + FragIT (2 µg/µL)
6. panitumumab-MMAE + FragIT (2 µg/µL)
7. panitumumab-DFO + FragIT (2 µg/µL)

Figure 3. LC/MS analyses of bevacizumab-MMAE scFc and F(ab')$_2$ fragments. The top panel shows the total ion chromatogram (TIC). The MS spectra of scFc fragments eluting at 5.12 min (bottom left) and 5.30 min (bottom right) are shown in the bottom panels.

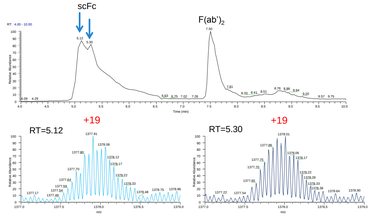


Figure 4. LC/MS analyses of panitumumab-MMAE scFc and F(ab')$_2$ fragments. The top panel shows the total ion chromatogram (TIC). The MS spectra of scFc fragments eluting at 4.46 min (bottom left) and 4.57 min (bottom right) are shown in the bottom panels.

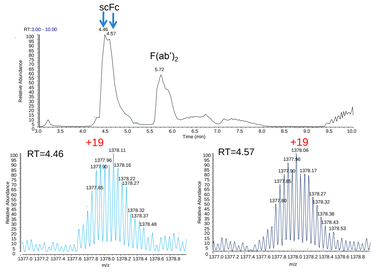


Figure 5. Deconvolution of bevacizumab scFc fragments before and after GlycINATOR cleavage and SiteClick labeling.

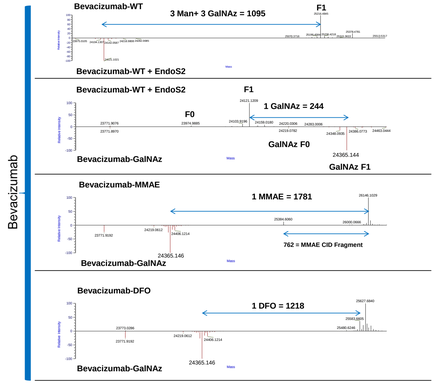


Figure 6. Deconvolution of panitumumab scFc fragments before and after GlycINATOR cleavage and SiteClick labeling.

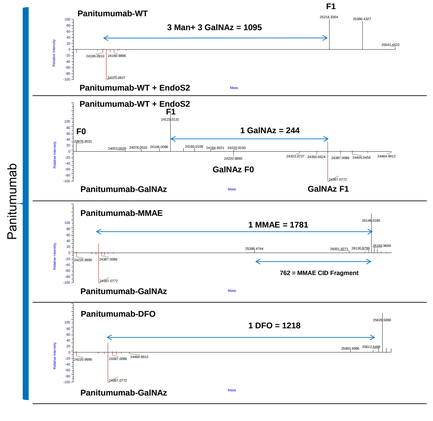
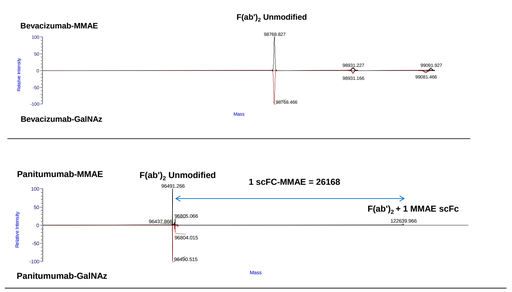


Figure 7. Deconvolution of bevacizumab and panitumumab scFc fragments before and after GlycINATOR cleavage and SiteClick labeling.



CONCLUSIONS

- SiteClick, a novel site-specific antibody labeling technology, can be applied to essentially any existing antibody with Fc-domain N-linked glycans.
- This antibody labeling technology has universal application and can be applied to multiple workflows from conjugating antibodies with small organic molecules to large fluorescent probes.
- An additional labeling approach using enzymatic pre-treatment, or "trimming" of the antibody glycans with GlycINATOR, prior to azide activation, enables the chemoenzymatic synthesis of homogeneous antibody conjugates with a degree of labeling of 2 labels per antibody.
- High resolution separation of mAb fragments scFc, scFc-MMAE, scFc-DFO, and F(ab')$_2$ is achieved using the MAbPac RP column.
- High resolution MS analysis of scFc fragments and its derivatives leads to successful characterization of site-specific ADCs.

REFERENCES

1. Site-specifically labeled CA19.9-targeted immunoconjugates for the PET, NIRF, and multimodal PET/NIRF imaging of pancreatic cancer. Houghton JL, Zeglis BM, Abdel-Atti D, Aggeler R, Sawada R, Agnew BJ, Scholz WW, Lewis JS. Proc Natl Acad Sci U S A. 2015 Dec 29;112(52):15850-5.

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