Comprehensive Characterization of Cysteine-conjugated Antibody Drug Conjugate (ADC) on a Hybrid Quadrupole-Orbitrap Mass Spectrometer

Xiaoxi Zhang[1], Kristina Srzentić[2] and Min Du[2], Thermo Fisher Scientific 1 Shanghai, China; 2 Cambridge, Massachusetts, US

ABSTRACT

Purpose: In-depth characterization of an antibody cysteine-fluorophore conjugate standard on a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer.

Methods: LC-MS based native/denatured intact mass analysis and peptide mapping.

Results: The molecular weights of this ADC were measured by intact mass analysis under native and denatured condition. The average DAR was automatically calculated by the software; drug conjugated site and linker only form were identified by peptide mapping.

INTRODUCTION

Antibody drug conjugates (ADCs) are considered one of the most promising types of biotherapeutics for cancer treatment. Comprehensive characterization and monitoring of their quality attributes is crucial during their development and manufacturing. In this study, an Orbitrap Exploris 240 mass spectrometer was used for in-depth characterization of SigmaMAb antibody drug conjugate (ADC) Mimic, an antibody cysteine-fluorophore conjugate standard. High performance native and denatured intact/subunit mass analysis as well as peptide mapping analysis were applied to assess the critical quality attributes (CQAs) of ADCs, including drug-to-antibody ratio (DAR), drug distribution, drug conjugation sites, and conjugate site occupancy.

MATERIALS AND METHODS

Sample Preparation

For peptide mapping, the sample was reduced by Dithiothreitol (DTT), followed by iodoacetic acid (IAC) treatment and then digested with trypsin.

For denature and native intact mass, the sample was diluted using ddH_2O to 1 mg/mL.

Test Methods

The denatured intact and peptide samples were separated on a Thermo Scientific™ Vanquish™ UHPLC system (A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile). A MAbPac™ RP column (4 µm, 2.1 mm x 100 mm, P/N 088647) was used for denatured intact MS and an Acclaim Vanquish C18 column (120Å; 2.2 µm, 2.1 x 150 mm, P/N 071399-V) for peptide mapping.

Native intact sample was separated on the same system using a MAbPac™ SEC-1 column (5 µm, 2.1 mm x 150 mm, P/N 088790) and solvent was 50mM ammonium acetate (30℃, 90 µL/min).

Different gradients and LC conditions were used for each analysis (Tables 1 and 2) on a Thermo Scientific™ Vanquish™ UHPLC system. An Orbitrap Exploris 240 MS was used for all analyses (Table 3).

Data Analysis

Data analysis was performed using Thermo Scientific™ Biopharma Finder™ software.

Table 1. Chromatography gradient for denature intact MS.

Column temp.: 80℃ / Flow rate: 250 µL/min	
Time(min)	%B
0.0	25
1.0	25
9.0	35
10.0	80
11.0	80
12.0	25
20.0	25

Table 2. Chromatography gradient for peptide mapping.

Column temp.: 40℃ / Flow rate: 300 µL/min			
Time (min)	%B	Time (min)	%B
0.0	1	81.0	1
5.0	1	83.5	10
6.0	10	91.5	45
70.0	60	93.0	90
72.0	90	99.0	90
77.0	90	101.0	1
79.0	1	115.0	1

Table 3. MS settings.

	Native intact	Denatured intact	Peptide mapping
Resolution@m/z=200	60K	30K	MS1 120K/MS2 15k
Mass range (m/z)	2500-8000	800-5000	MS1 200-2500
Application mode	Intact protein(high pressure)	Intact protein(high pressure)	Peptide mapping(standard)
Fragmentation	In-source CID 120	In-source CID 60	HCD 27%

RESULTS

Intact mass analysis under native conditions

The diverse nature of ADC components is due in part to chemical modifications which arise from the manufacture of the core antibody as well as the drug conjugation (Figure 1). Major forms of cysteine-linked ADCs differ in N-glycan composition, the number of linker-drugs attached, as well as potential linker-only attachments.

Figure 1. Schematic of the Sigma ADC Mimic.

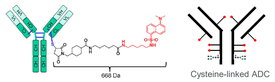


Figure 2. Native size exclusion chromatography (SEC) coupled to mass spectrometry for the analysis of the SigmaMAb ADC mimic. (A) Base peak chromatogram. (B) Orbitrap full MS spectrum acquired on Orbitrap Exploris 240 with BioPharma Option at R=60,000. (C) An expanded view of the 27+ charge state. (D) the ReSpect™ deconvolution results, showing a distribution of 0-8 SMCC linker-drug attachments. Average Drug-to-Antibody Ratio (DAR) was calculated by software automatically, based on all identified glycoforms.

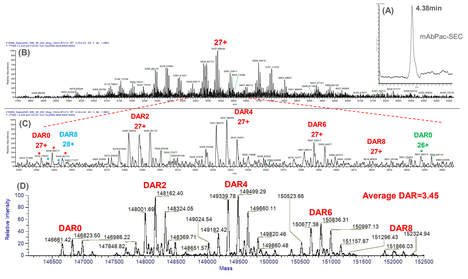


Table 4. Mass accuracy and relative abundance of the most abundant glycoform (G0F/G1F).

G0F+G1F DAR	Avg. Mass (Da)	Mass Accuracy(ppm)	Rel. Abundance%
DAR0	146823.50	1.2	26.58
DAR2	148162.40	6.5	96.60
DAR4	149499.29	0.4	100.00
DAR6	150836.31	6.3	52.84
DAR8	152170.75	4.0	6.66

Table 5. All possible conjugated sites of Sigma ADC Mimic.

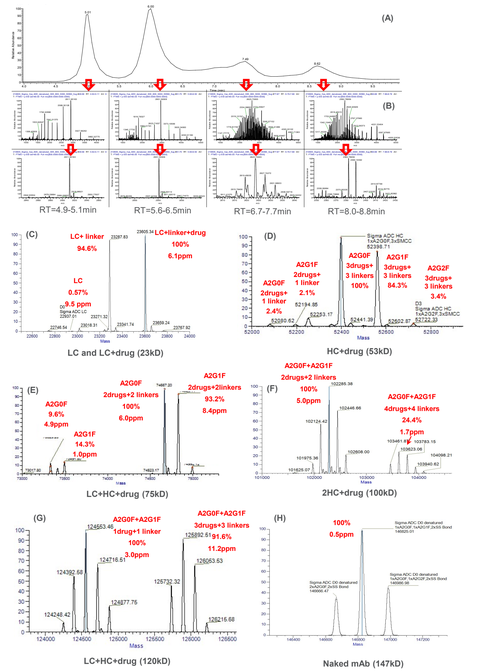
Site	Peptide sequence	Site occupancy
LC-C217	TVAPTE**C**S	Carboxymethylation Drug-linker Linker only
HC-C224	S**C**DK	Carboxymethylation Drug-linker Linker only
HC-C230,233	THT**C**PPCPAPELLGGPSVFLFPPKPK	2 carboxymethylations 1 drug-linker +1 carboxymethylation 2 drug-linkers 1 drug-linker +1 linker only 2 linker only

Denatured Intact mass analysis

The inter chain disulfide bonds of cysteine-linked ADCs are reduced for link-drug attachment. Therefore, the non-covalent bonding between light chain and heavy chain will be broken down under denatured conditions. Multiple ADC related species(Figure 3) can be observed in denatured intact mass results(Figure 4). Very accurate intact masses for the species are obtained (Figure 4, C-H).

Figure 3. Schematic of all possible species of Sigma ADC Mimic under denatured condition.

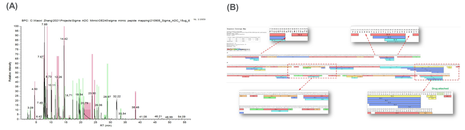
Figure 4. RP LC-MS intact mass analysis of Sigma ADC Mimic under denatured condition. (A) LC separation. (B) Full MS and zoom in spectra at 30k resolution for corresponding peaks. (C-H) the ReSpect deconvolution results.



Peptide mapping results

Sigma ADC Mimic digested peptide mixture was analyzed using RP LC-MS/MS and resulted in >93% sequence coverage as shown in Figure 5.

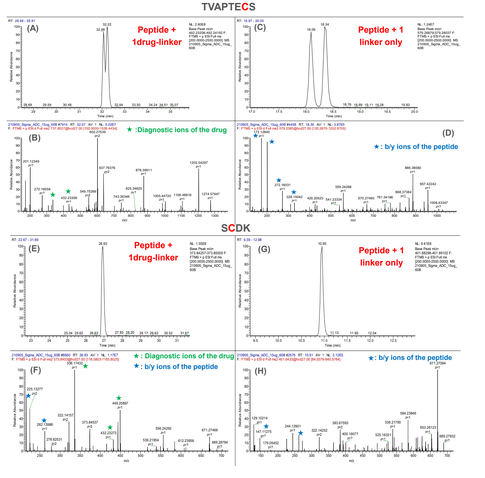
Figure 5. Sigma ADC Mimic peptide mapping results showing base peak chromatograms(A) and sequence coverage(B). It is noteworthy that trypsin digestion generated some small peptides which are too short to trigger MS² (red dotted frame insert).



The drug-linker attached peptide characterization is challenging because the chemical conjugation makes the peptide more hydrophobic compares to its unconjugated form. Also, the complexity increases because both drug-linker and linker only attached forms may exist in the same sample, especially for peptides contain multiple cysteines. Finally, the drug was fragmented under HCD collision. Therefore, we need to use both MS1 m/z and diagnostic MS2 ions generated by the drug to confirm the drug-linker or linker only attachment.

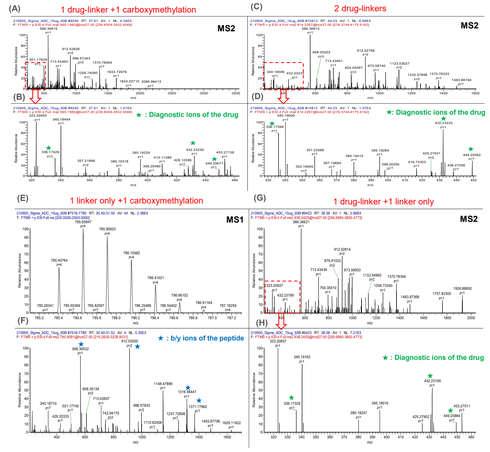
In this study, we successfully identified most of the conjugated peptides listed in table 5 except carboxymethylated SCDK and 2 linker only form of hinge region peptide. These sites may be highly occupied by drug-linker attachment, results in no detection of these two forms. Figure 6 shows the chromatogram peaks and MS2 spectra of peptides TVAPTECS and SCDK. Diagnostic MS2 ions generated by the drug were marked in the spectra.

Figure 6. drug-linker and linker only form of peptides TVAPTECS(A~D) and SCDK(E~H). A~B, chromatogram peaks and MS2 of drug-linker TVAPTECS. C~D, chromatogram peaks and MS2 of linker only TVAPTECS. E~F, chromatogram peaks and MS2 of drug-linker SCDK. G~H, chromatogram peaks and MS2 of linker only SCDK.



For hinge region peptide THTCPPCPAPELLGGPSVFLFPPKPK, in addition to the carboxymethylated form (data not shown), four different conjugated forms were identified (Figure 7). Diagnostic MS2 ions generated by the drug and b/y ions of peptide were marked in the spectra.

Figure 7. Different conjugated forms of peptides THTCPPCPAPELLGGPSVFLFPPKPK. A~B, MS2 of 1drug-linker +1 carboxymethylation form and expanded view. C~D, MS2 of 2 drug-linkers form and expanded view. E~F, MS1 of 1 linker only +1 carboxymethylation form and MS2 spectrum. G~H, MS2 of 1drug-linker +1 linker only form and expanded view.



CONCLUSIONS

- In this work, we performed native/ denatured intact mass analysis and peptide mapping of Cysteine conjugated ADC mimic on an Orbitrap Exploris 240 mass spectrometer.
- Successfully measured drug-to-antibody ratio (DAR) and drug distribution under native and denatured conditions.
- Drug conjugation sites and conjugate site occupancy were fully characterized using peptide mapping.

TRADEMARKS/LICENSING

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