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Measurement of Cysteine-linked ADCs Under Native Conditions Using an Orbitrap Mass Analyzer

Jing Li,[1] Shujun Yang,[2] Nianhe Han,[2] Li Jian[2]
[1]Thermo Fisher Scientific, Shanghai, China; [2]NewBio Therapeutics Inc, Shanghai, China

Overview

Purpose: Cysteine-linked Antibody-drug conjugates (ADCs) measurement was a great analytical challenge resulted from the presence of a mixture of covalent and noncovalently associated light chain and heavy chain subdomains. A native mass spectrometry(MS) methodology based on Thermo Scientific™ Exactive™ Plus EMR Orbitrap™ mass spectrometer was evaluated for cysteine -linked ADCs measurement.

Methods: After deglycosylation with PNGase F and buffer exchange with 100 mM ammonium acetate (pH 7.0), One cysteine -linked ADCs drug under research (PCT) was introduced using NanoFlex static nanospray source onto Exactive Plus EMR MS. Data processing was performed on Thermo Scientific™ Protein Deconvolution™ software.

Results: For each set of peaks of ADCs with various payloads, they were resolved from their adducts with the increase of resolution setting from 17,500 to 35,000.The mass accuracy also greatly improved with the increase of resolution. Thus drug-to-antibody ratio(DAR) can be determined accurately. Relative ratios of each detected compound were determined using MS peak intensities and served to estimate the average DAR (3.9),which is consistent with the detected DAR from hydrophobic interaction chromatography (HIC).

Introduction

ADCs are biochemotherapeutics constituted of a cytotoxic chemical drug linked covalently to a monoclonal antibody(mAb). Drug conjugation can be achieved via reactions at different amino acid residues or glycans: at lysine side chains amines, at cysteine thiol groups after reduction of the interchain disulfide bonds, or at engineered cysteine residues at specific sites in mAb without disruption of interchain disulfide bonds. Generally, the average DAR value, the drug load distribution need to be tightly controlled for ADCs. For the prevalent family of cysteine-linked ADCs generated by partial reduction of the antibody interchain disulfides prior to conjugation, an additional analytical challenge results from the presence of a mixture of covalent and noncovalently associated light chain and heavy chain subdomains[1] (Figure 1). Analytical methods should prevent disrupting the noncovalent associations between ADC subdomains.

Native MS has emerged as a valuable technique for characterization of intact noncovalent protein complexes, reaching a high level of reliability within the last ten years[2]. Native MS allows to retain noncovalent structures, which can be used in cysteine-linked ADCs profiling.

The Exactive Plus EMR mass spectrometer combines unsurpassed high-resolution accurate-mass Thermo Scientific™ Orbitrap™ analysis with an extended mass range (EMR) option to create an outstanding tool for investigating the structure, topology, and architecture of native-like tertiary and quaternary protein structures. It enables the accurate measurement of DAR and the drug load distribution of cysteine-linked ADCs under their native conditions[3].

Methods

Sample Preparation

The intact cysteine-linked ADCs (PCT) was deglycosylated using PNGase F(NEB). Then this sample was buffer exchanged against 100 mM ammonium acetate (AcONH4) pH 7.0 with Micro Bio-Spin chromatography columns (Bio-Rad). ADCs mixture was injected at 5 µM on the Exactive Plus EMR Orbitrap mass spectrometer.

Mass Spectrometry

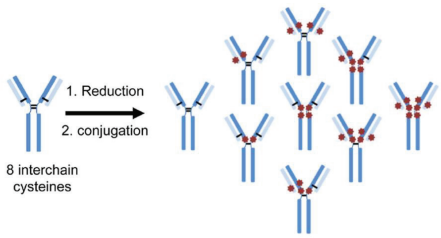
Infusion condition	
Instrumentation	NanoFlex Source with emitter
Ionization voltage (kV)	1.5
Polarity	Positive

MS condition	
Instrumentation	Exactive Plus EMR Orbitrap MS system
EMR mode	ON
Mass range (m/z)	3000–10000
Resolution	17,500 35,000 @ m/z 200
Target value	3 x 10^6
Max injection time (ms)	300
Microscans	10
Insource CID energy (eV)	175
S-lens level (%)	200
HCD energy (eV)	25
Spectra average	50

Data Processing

Data Processing	
Software	Protein Deconvolution
Deconvolution parameters	
Noise compensation	ON
Minimum adjacent charges	1 to 3
Noise Rejection	95% confidence

FIGURE 1. Schematic representation of cysteine-linked ADCs formation



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Results

Raw Data Acquisition

The intact cysteine-linked ADCs (PCT) was analyzed on the Exactive Plus EMR MS with resolution set at 17,500, 35,000. The most intense charge envelope was between m/z 5000 to m/z 7500 under native conditions, which differed from charge envelope distribution under classic denaturing conditions . Figure 2 shows the raw mass spectra with entire charge state distribution of this ADCs under native conditions. Zoom of the m/z regions 6110-6175 was shown in Figure 3, for each set of peaks of ADCs with various payloads, they were resolved from their adducts with the increase of resolution setting from 17,500 to 35,000.

FIGURE 2. Orbitrap native raw mass spectra of PCT with resolution set at 17,500, 35,000.

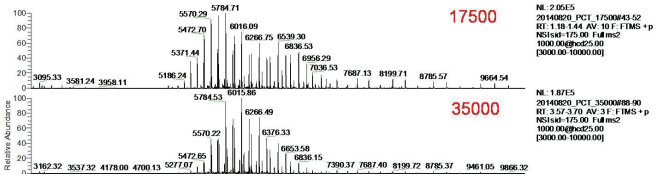
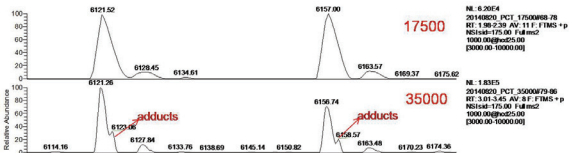


FIGURE 3. Orbitrap native raw mass spectra of PCT with resolution set at 17,500, 35,000 (m/z regions 6110-6175)



Deconvoluted Data

These raw files acquired at various resolution were processed with Protein Deconvolution software. As shown in Figure 4, populations with zero, two, four ,six and eight molecules loaded onto the antibody (payloads) were detected with a mass difference between peaks corresponding to the addition of two payloads (a mass increase of +2,635Da ,Table 1). It was proved that native MS enabled easy detection of drug load heterogeneity, providing an instantaneous snapshot of the drug-load distribution.

As observed in Table 1, better mass accuracies were obtained with the increase of resolution setting from 17,500 to 35,000.This phenomenon can be attributed to the well-resolved peaks of ADCs from their adducts since nonspecific interactions of metal adduct ions such as sodium, potassium, and so on are very common under native conditions. Though removal of adduct ions can be performed offline by ultrafiltration or spin column, however, these ADCs still retained partial adducts, which attached great importance to high resolution and mass accuracies in Native MS.

FIGURE 4. Orbitrap native raw mass spectra of PCT with resolution set to 17,500, 35,000.

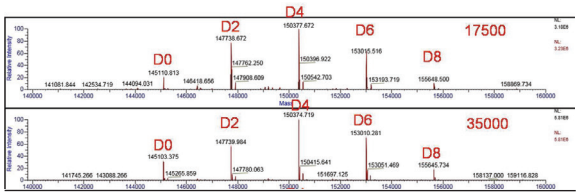


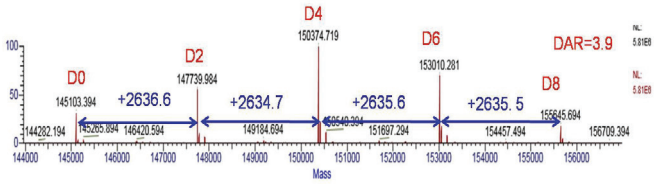
TABLE 1. Masses measured in Native MS for PCT under 17,500, 35,000 resolution

Resolution	No.of Drug Load	Measured Mw（Da）	Theoretical Mw（Da）	Delta M (Da)	Mass Accuracy (ppm)
17500	D0	145111	145103	7.7	53.1
	D2	147739	147738	0.3	2.0
	D4	150378	150374	4.1	27.3
	D6	153016	153009	6.6	43.1
	D8	155649	155644	4.4	28.3
35000	D0	145103	145103	0.3	2.1
	D2	147740	147738	1.6	10.8
	D4	150375	150374	1.1	7.3
	D6	153010	153009	1.4	9.1
	D8	155646	155644	1.6	10.3

Determination of DAR

Relative ratios of each detected compound were determined using MS peak intensities and served to estimate the DAR, Average DAR value was obtained by summing up the weighted peak percentage from all observed species and dividing the sum by 100, as follows: DAR = Σ(relative peak area×number of loaded drugs)/100(number of drug load ranging from 0 to 8). For example, DAR was calculated as 3.9 for raw data detected under 35,000 resolution(Figure 5), which was consistent with the detected DAR from HIC (Data not shown).

Figure 5. Deconvoluted spectra of PCT detected at resolution 35,000



Conclusion

- In this analysis using the Exactive Plus EMR MS, molecular weight measurements of cysteine-linked ADCs in the low ppm mass deviation range allowed the identification of all species of ADCs.
- The Exactive Plus EMR MS was able to sensitively characterize ADC complexes with mass differences between peaks corresponding to different additional number of payloads/drugs.
- Benefit of improved mass accuracy capabilities of high resolution native MS was highlighted for the determination of ADC's drug load profile and naked antibody.
- For each set of peaks, the DAR can be determined as well as the relative ratio of each detected species.

References

1. Debaene, F. *et al.*, Innovative native MS methodologies for antibody drug conjugate characterization: High resolution native MS and IM-MS for average DAR and DAR distribution assessment. *Anal Chem* 2014,86(21):10674-83.
2. Albert J R Heck *et al.* Native mass spectrometry: a bridge between interactomics and structural biology. *Nat. Methods* 2008,5(11), 927-933.
3. Yue X *et al.* Monoclonal Antibody and Related Product Characterization Under Native Conditions Using a Benchtop Mass Spectrometer. *Thermo Fisher Poster*, 2014, ASMS.