Imaged capillary isoelectric focusing – Mass Spectrometry (iCIEF- MS) online coupling for polatuzumab vedotin charge heterogeneity analysis using native MS

Xiaoxi Zhang[1], Tony Chen[2], Tao Bo[2], Tiemin Huang[2], Min Du[3]. 1 Thermo Fisher Scientific, Shanghai, China, 2 Advanced Electrophoresis Solutions Ltd, Cambridge, Canada,
3 Thermo Fisher Scientific, Lexington, MA, US

Abstract

Purpose: To demonstrate the capability of iCIEF-MS online coupling of cysteine-linked ADC charge variant analysis under near native condition.

Methods: CEinfinite iCIEF platform and Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometry with Biopharma option were employed.

Results: Successfully separated and identified charge variants of polatuzumab vedotin, a latest-generation cysteine-linked ADC using iCIEF-MS online coupling platform under native condition.

Introduction

Antibody-drug conjugates (ADCs) represent the forefront of the next generation of biopharmaceuticals. An ADC typically comprises an antibody covalently linked to a cytotoxic drug via a linker, resulting in a highly heterogeneous product. The conjugation will bring extra charge heterogeneity to the biomolecule, therefore, charge based separation technologies are important in ADC characterization.

iCIEF has become an indispensable tool in therapeutic protein development and manufacturing because of its high analytical throughput, ease of use, fast method development and excellent reproducibility. Recently, iCIEF-MS has attracted much attention to utilize for protein charge variant analysis. Here we did online iCIEF-MS to analyze charge heterogeneity of polatuzumab vedotin(Polivy®, Roche), a latest-generation cysteine-linked ADC, using CEinfinite iCIEF platform and Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometry.

Materials and methods

Sample Preparation

Commercially available polatuzumab vedotin was dissolved in ddH_2O and desalted using 10k cut-off filter. Final protein concentration is 1.5mg/mL in ddH_2O with 1%HR3-10 and 1%HR6-8 carrier ampholytes.

iCIEF Focusing and mobilisation

200 µm ID acrylamide derivative coated (AD) capillary cartridge was used. The focusing was performed using 1 min-1000 V, 1 min-2000 V and 10 min-3000 V. 3000 V was applied during mobilisation of focused protein bands; the mobilisation speed was 70 nL/min with 0.1% FA, H_2O and 3 µL/min make up solution (10mM NH4Ac:ACN=9:1) added through a micro tee. Mobilisation time was 50 min.

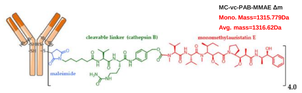
Mass Spectrometry:

An Orbitrap Exploris™ 240 mass spectrometry with Biopharma option was used for data acquisition. Both iCIEF and MS platforms were controlled using Thermo Scientific™ Chromeleon™ software.

Data Analysis

Data analysis was performed using Thermo Scientific™ BiopharmaFinder™ software 5.2.

Figure 1. The Schematic of polatuzumab vedotin.



Results

Method optimization for iCIEF-MS online coupling under near native condition

Polatuzumab vedotin, a latest-generation cysteine-linked ADC was used in this study. The schematic of polatuzumab vedotin is shown in Figure1[1]. During the conjugation of polatuzumab vedotin, the interchain disulfide bridges of mAb were partially reduced, and the mAb was bioconjugated to a maleimidocaproyl-valinecitrulline-p-aminobenzyloxycarbonyl linker- monomethyl auristatin E (MC-vc-PAB-MMAE), leads to a 0/2/4/6/8 payload distribution. The average DAR of this ADC is around 4 according to previous publication[1]. As the interchain disulfide bridges of the mAb were reduced for conjugation, the light and heavy chains (LC and HCs) are non-covalently bonded. Therefore, native intact MS is essential for charge heterogeneity analysis of polatuzumab vedotin at the intact ADC level.

Figure2 illustrates iCIEF-MS online coupling workflow. After proteins are separated into groups based on their isoelectric point (pI), the subsequent mobilization process introduces protein bands into MS to isolate the charge isomer peaks. During pressure mobilization, an electric field keeps the samples in the separation capillary focused. Thermo Scientific™ Chromeleon™ software can control CEinfinite iCIEF platform and Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometry for data acquisition, which better meets compliance requirements.

Figure 2. iCIEF-MS online coupling workflow.

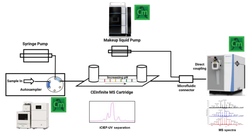
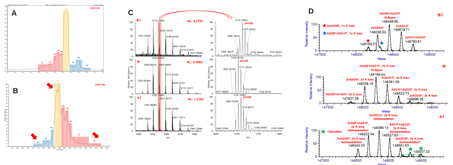


Table 1. MS settings.

Source parameters		Full scan parameters	
Sheath gas	0	SID	120eV
Aux gas	5	Microscans	10
Spray voltage	3.8kV	Resolution	30,000
Capillary temp.	250°C	Scan range	2500-8000
RF-lens	200	Application mode	Intact Protein
Aux gas temp.	40 °C	Pressure mode	High pressure

iCIEF-MS online coupling under denaturing condition is a mature workflow now and has been widely used in mAb and fusion protein charge variant analysis[2-6]. However, iCIEF-MS online coupling under native condition is quite challenging because the MS signal intensity of protein is much lower under native condition compares to denaturing condition, which means the sensitivity of MS is critical, especially for low abundance charge variants identification. Also, the make-up liquid needs changing to MS compatible buffer and keep samples stay native. Figure3 shows iCIEF-MS online coupling under native condition of NISTmAb charge variant analysis. It is clearly that charge isomer peaks were well separated by iCIEF, benefitted from highly sensitivity of Orbitrap analyzer, even the low abundance basic peak (B3) components had high S/N in raw MS spectra. The mass accuracy of A2G0F+A2G1F, 2x K loss in main peak is 0.9ppm, proved the mass accuracy of Orbitrap platform.

Figure 3. iCIEF-MS online coupling of NISTmAb under native condition, 1.6µg sample was loaded. A, iCIEF-UV profile. B, iCIEF-MS profile. C, raw spectra of basic peak3(B3), main peak(M) and acidic peak1(A1), as labelled in 3B. D, deconvoluted spectra of B3, M and A1.



As discussed in previous section, to keep the protein in native state, the make-up liquid and mobilisation liquid composition are critical. For native mAb analysis, the combination of make-up liquid (10mM NH4Ac:ACN=9:1) and mobilisation liquid (0.1%FA, H_2O) can maintain mAb at native condition, as shown in Figure3. However, when preparing ADC sample for iCIEF analysis, a common approach is to add formamide into sample solution to improve solubility. But formamide will break the non-covalently bonds between chains of cysteine-linked ADC. Figure4 displays iCIEF-UV profile and MS spectra of main peak, with 0%, 1% and 5% formamide in polatuzumab vedotin (v/v) respectively. It is clearly that formamide leads to better peak shape and separation at iCIEF level, but even with lower to 1% formamide, the LC/HC dissociation can be observed in MS spectra. To keep the ADC sample at native state, we choose no formamide in ADC sample as the final condition.

Figure 4.sample buffer optimization for polatuzumab vedotin.

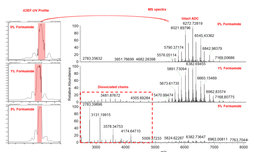
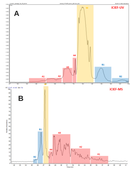


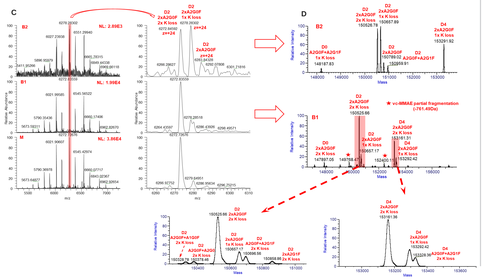
Figure 5. A and B.



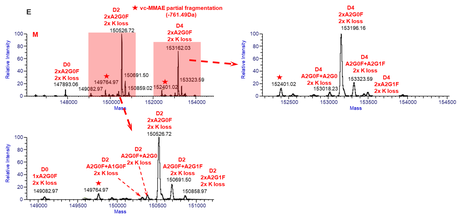
iCIEF-MS online coupling of polatuzumab vedotin under near native condition

iCIEF-MS online coupling of polatuzumab vedotin was performed with optimized experiment make-up liquid (10mM NH4Ac:ACN=9:1), mobilisation liquid (0.1%FA, H_2O) and no formamide in sample. Figure5 A and B display iCIEF-UV and iCIEF-MS profiles, four acidic peaks, main peak and two basic peaks were separated and identified. iCIEF-UV and iCIEF- MS profiles are mirror symmetric because the basic peaks at right were introduced to MS source at first.

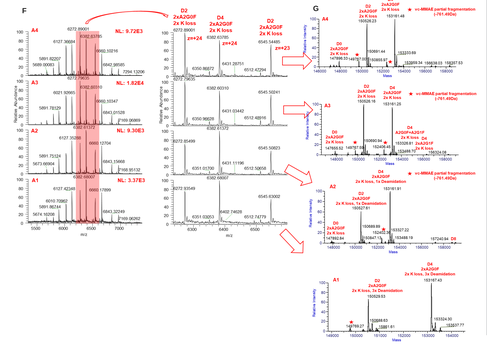
Figure 5. iCIEF-MS online coupling of polatuzumab vedotin under native condition, 2.4µg sample was loaded. A, iCIEF-UV profile. B, iCIEF-MS profile. C, raw spectra of basic peak1-2(B1-2) and main peak(M). D, deconvoluted spectra of B1 and B2. E, deconvoluted spectra of main peak. F, raw spectra of acidic peak1-4(A1-4). G, deconvoluted spectra of acidic peak1-4(A1-4).



Without formamide in sample, we lost separation resolution at iCIEF level (Figure5A), but Orbitrap based MS platform could provide high resolution to compensate the loss. Another advantage of Orbitrap MS platform is excellent sensitivity. In this experiment, only 2.4µg sample was loaded, and compares to denaturing condition, the MS signal of intact protein is much lower under native condition. Even for low abundance basic peak2 (relative abundance 0.79%), the S/N of raw spectra is high (Figure5C). D2 and D4 carrying one or two lysine at C-terminal of heavy chain were identified in B2 (Figure 5D). B1 is mixture of D2 and D4 with 2x K loss or 1x K loss. The N-glycosylation distribution of D2 and D4 were shown in expanded view of Figure5D. Low abundance N-glycoforms, such as A2G0F+A1G0F and A2G0F+A2G0 were detected. Vc-MMAE partial fragmentation mass shift (-761.49Da) was observed and labelled with red star.



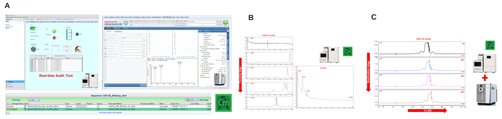
The major components in main peak are D2 and D4 N-glycosylation distribution with 2x K loss (Figure5E). Figure5F displays the raw spectra of acidic peaks. In acidic peak A4 and A3, D2/D4 ratio varied while components are almost the same; the deamidated variants were detected in acidic peak A2 and A1. With increasing pI, the relative abundance% of D4 increased. In basic and main peak, the top drug payload isoform is D2 while in acidic peak A4, A2 and A1, D4 became the top drug payload isoform, indicates drug conjugation may affects charge heterogeneity of ADC, especially the net charge distribution. It looks like iCIEF separates charge variants of cysteine-linked ADC based on PTM-induced charge heterogeneity more than bioconjugation-induced heterogeneity.



Chromeleon control for both CEinfinite iCIEF platform and Orbitrap Exploris™ 240 mass spectrometry

The driver of CEinfinite iCIEF platform has been already integrated into Chromeleon, which means Chromeleon can be used for CEinfinite and multiple Orbitrap series MS instrument control now, including instrument calibration and data acquisition. Figure6A shows Chromeleon software page, including CEinfinite iCIEF platform page, Thermo MS tuning page and running sequence. Both real-time and historical audit trails log can be read or exported according to user's demand. Figure6B is iCIEF-UV profiles and current channels during focusing stage and Figure6C is iCIEF-UV profiles during mobilisation stage. All information were recorded in Chromeleon data file, which could be checked or transferred conveniently in compliance-ready environment.

Figure 6. Chromeleon control for both CEinfinite iCIEF platform and Orbitrap Exploris™ 240 mass spectrometry . A, software interface. B, iCIEF-UV profiles and current channels during focusing stage. C, iCIEF-UV profiles during mobilisation stage.



Conclusions

- Successfully demonstrated the iCIEF-MS online coupling workflow for polatuzumab vedotin charge variant analysis under near native condition, charge variants of this cysteine-linked ADC were separated by iCIEF and identified by HRAM MS subsequently. iCIEF separates charge variants of cysteine-linked ADC based on PTM-induced charge heterogeneity more than bioconjugation-induced heterogeneity.
- Validated the feasibility of Chromeleon control for both CEinfinite iCIEF platform and Orbitrap Exploris™ 240 mass spectrometry.

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