Characterization of monoclonal antibodies and antibody drug conjugates using iCIEF-MS online coupling platform

Xiaoxi Zhang[1], Tiemin Huang[2], Tony Chen[2], Tao Bo[3], Min Du[4]. 1 Thermo Fisher Scientific, Shanghai, China, 2 Advanced Electrophoresis Solutions Ltd., Cambridge, Ontario, Canada, 3 Thermo Fisher Scientific, Beijing, China, 4 Thermo Fisher Scientific, Massachusetts, US

ABSTRACT

Purpose: To demonstrate the capability of iCIEF-MS online coupling platform for separation and real-time intact protein analysis of charge variants of mAb and ADC.

Methods: A CEInfinite iCIEF coupled with HRAM Orbitrap mass spectrometer was used for analyzing charge variants at intact level.

Results: Successfully separated and measured the molecular weight of mAb and ADC charge variants.

INTRODUCTION

Characterization and quantification of biopharmaceutical charge variants are required for assessing and ensuring product quality. Biopharmaceutical scientists routinely rely on Capillary IsoElectric Focusing (CIEF) and imaged CIEF (iCIEF) to assess the charge heterogeneity of monoclonal antibodies (mAbs). It is also a powerful tool to separate and identify antibody-drug conjugate (ADC) with different drug load based on their charge heterogeneity. Furthermore, the direct coupling of iCIEF to a Mass Spectrometer (MS) for characterization would accelerate the characterization process significantly.

In this study, a CEInfinite iCIEF coupled with an HRAM Orbitrap mass spectrometer was used for analyzing charge variants of pembrolizumab and T-DM1, an ADC consisting of the humanized mAb trastuzumab covalently linked to the cytotoxic drug DM1.

MATERIALS AND METHODS

Sample Preparation

mAb: HR AESlyte 6-8 (carrier ampholytes) was added into commercially available pembrolizumab (25mg/mL) and diluted using ddH_2O. Final protein concentration is 1mg/mL and 2% ampholytes(HR AESlyte 6-8).

ADC: commercially available T-DM1 was dissolved in ddH_2O with HR AESlyte 3-10, HR AESlyte 8.5-9.5 (carrier ampholytes) and formamide added. Final protein concentration is 1mg/mL, 4% ampholytes(2%HR AESlyte 3-10, 2%HR AESlyte 8.5-9.5) and 10% formamide.

iCIEF Separation

AES cartridge (ID200µm) was used for separation, iCIEF settings are listed in Table 1.

Mass Spectrometry

A Thermo Scientific™ Q Exactive™ Plus BioPharma mass spectrometer was used for all analysis. All settings are listed in Table 2.

Data Analysis

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

Table 1. iCIEF parameters

Sample	Pembrolizumab	T-DM1
Sample amount	~1.6µL/1.6µg per run	
Focusing	1500V-1min, 3000V-10min	1000V-1min, 3000V-9min
Mobilization	3000V-45min	
Mobilization liquid	10mM acetic acid, 40nL/min	0.1% formic acid (pH=2.5), 50nL/min
Make-up liquid	50%ACN,0.1% formic acid, 5µL/min	

Table 2. MS parameters

Pembrolizumab and T-DM1	
Sheath gas: 10Arb	In-source CID: 70eV
Aux gas: 0 Arb	S-lens: 60
Spray voltage: 3.9kV	Microscans: 10
Capillary temp.: 275℃	Resolution: 35,000
Aux gas temp.: 100 ℃	Scan range: 1500-5000

RESULTS

iCIEF separation and real-time intact protein analysis of pembrolizumab charge variants

Pembrolizumab is a highly selective anti-PD-1 humanized mAb with multiple charge variants. In this study eight peaks were successfully separated, including four acidic peaks, three basic peaks and main peak for subsequent online intact protein analysis by mass spectrometry. The online coupling schematic diagram is shown in Figure 1. The iCIEF profile is shown in Figure 2 and it is easily to find out that the separation is pretty good, even for very close peaks.

Figure 1. iCIEF-MS online coupling schematic diagram. Cartridge column out is directly connected to MS source without extra interface.

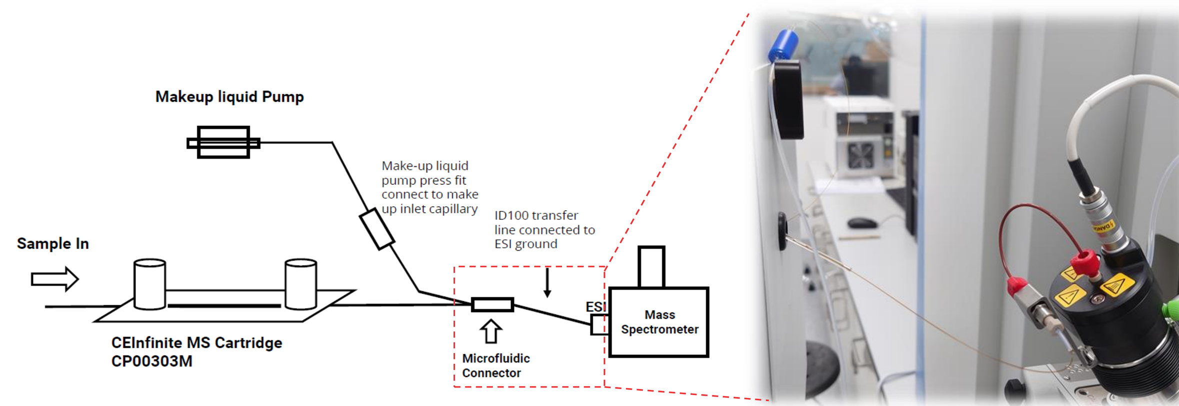
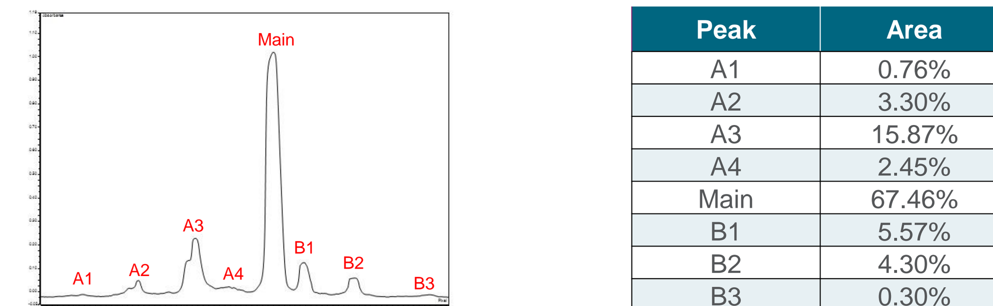


Figure 2. iCIEF- UV profile of pembrolizumab. About 1.6µg sample was loaded on the column. A1-A4, acidic peaks. Main, main peak. B1-B3, basic peaks.



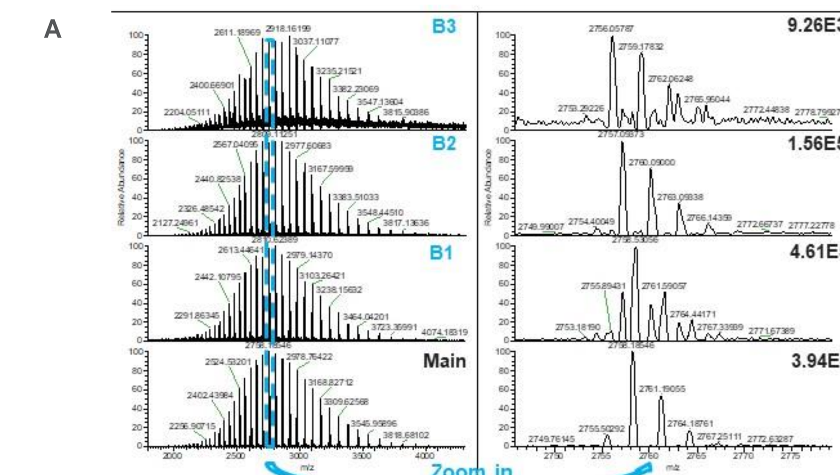
Peak	Area
A1	0.76%
A2	3.30%
A3	15.87%
A4	2.45%
Main	67.46%
B1	5.57%
B2	4.30%
B3	0.30%

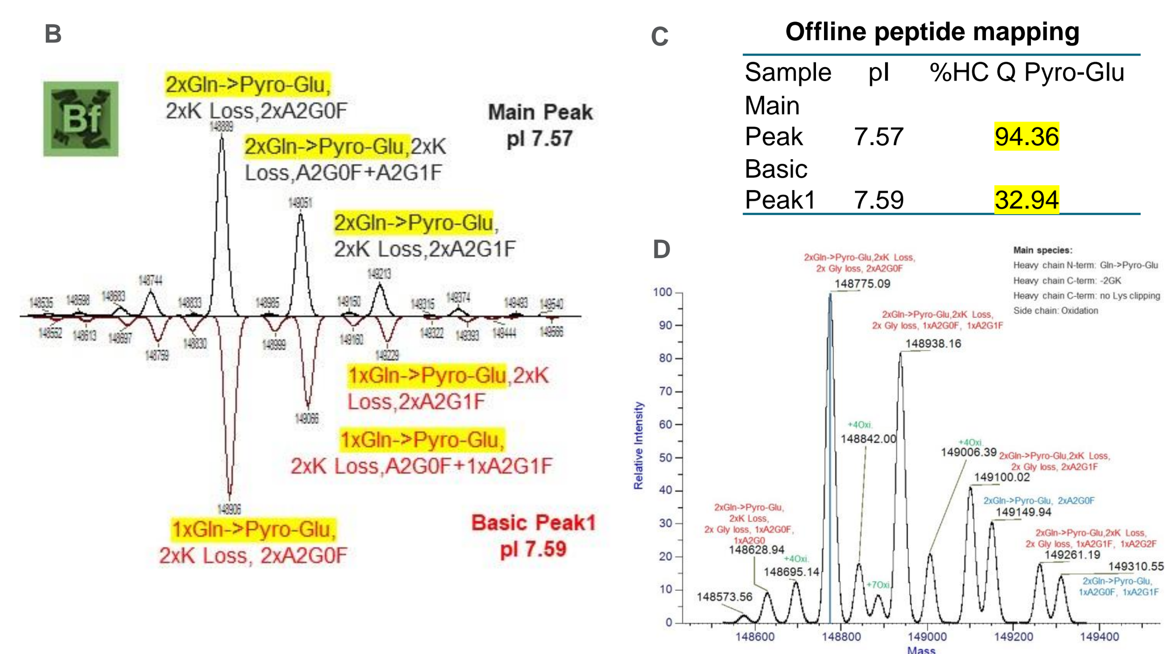
Benefitting from the high sensitivity of Orbitrap mass spectrometry, trace variants, like Basic peak B3 (~0.3% of all peaks, ~4.8ng) can be detected with good signal to noise ratio. Figure 3A depicts the comparison of the raw spectra of basic peaks and main peak. It is easy to observe mass shift even at the raw spectrum level because basic variants are mainly caused by heavy chain C-terminal truncation, heavy chain N-terminal pyroglutamate cyclization and oxidation.

Figure 3B is the mirror plot of main peak (pI=7.57) and basic peak1 (pI=7.59) deconvolution results. A series of 17 Da mass shifts can be detected for every glycoform. The reason is that compared to the main peak, only one glutamine at N-terminal of heavy chain was cyclized to form pyroglutamate, and this intact protein analysis results corresponds with the previous peptide mapping result [1] (Figure 3C).

Figure 3. iCIEF- MS online coupling results of pembrolizumab charge variants.

A, raw spectra comparison of basic peaks and main peak. B, the mirror plot of main peak (pI=7.57) and basic peak1 (pI=7.59) deconvolution results. C, The Q pyro-glu% measured by previous offline peptide mapping result. D, deconvolution result of basic peak3 (B3).





All charge variants identification results are summarized in Table 3. For basic peaks, the main PTM caused charge heterogeneity are heavy chain C-terminal truncation, heavy chain N-terminal pyroglutamate cyclization and oxidation. All the modification details could be clearly read out in the MS intact results. For example, -GK truncation (-185Da) was identified on C-terminal of both heavy chains in the lowest basic peak (B3), which is two orders lower than main peak (Figure 3D). In acidic peaks, series of deamidation and different sialic acid modifications were identified.

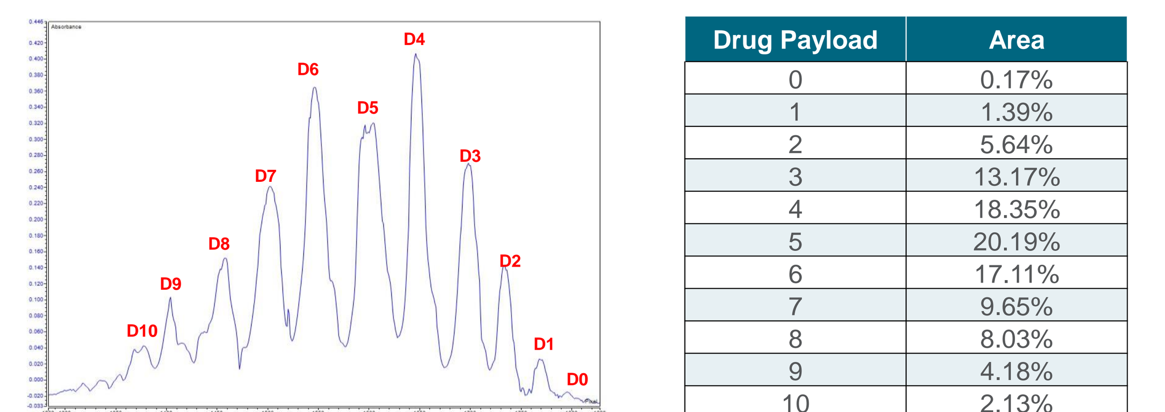
Table 3. All charge variants identification results of pembrolizumab. HC=heavy chain.

Peak	Main	B1	B2	B3
Modifications	HC N-term: Gln->Pyro-Glu HC C-term: -2K	HC N-term: Gln->Pyro-Glu(~30%) HC C-term: -2K	HC N-term: Gln->Pyro-Glu HC C-term: -1GK&-1K HC C-term: one Lys clipping Side chain: Oxidation	HC N-term: Gln->Pyro-Glu HC C-term: -2GK HC C-term: no Lys clipping Side chain: Oxidation
Peak	**A1**	**A2**	**A3**	**A4**
Modifications	Heavy chain N-term: Gln->Pyro-Glu Heavy chain C-term: -2K Side chain: Deamidation, sialic acid			

iCIEF separation and real-time intact protein analysis of T-DM1 different drug payload forms

Trastuzumab emtansine, which is also called T-DM1, is an antibody-drug conjugate consisting of the humanized monoclonal antibody trastuzumab (Herceptin) covalently linked to the cytotoxic agent DM1. For this lysine conjugated ADC, different drug loads from D0 to D10 were clearly separated at iCIEF-UV level (Figure 4). This represents a meaningful result, because these isoforms cannot be separated on routine RPLC-MS.

Figure 4. iCIEF- UV profile of T-DM1. About 1.6µg sample was loaded on the column. D0-D10, different drug payloads.



Drug Payload	Area
0	0.17%
1	1.39%
2	5.64%
3	13.17%
4	18.35%
5	20.19%
6	17.11%
7	9.65%
8	8.03%
9	4.18%
10	2.13%

ADCs are complex molecules composed of an antibody linked to a biologically active cytotoxic (anticancer) payload or drug, so charge-based separation before intact MS analysis can significantly reduce the complexity of spectra, making interpretation easier.

We compared the MS spectra and deconvolution results of RPLC-MS and iCIEF-MS and found out that using the iCIEF-MS strategy, separation of ADC with different drug loads reduced the interference of MS signals from adjacent charge states and drug payloads (Figure 5-9). Therefore, the deconvolution results were simplified.

Figure 5. iCIEF- MS spectra of T-DM1. A, D0 to D10 (simulation of no iCIEF separation condition). B, D0. C, D2. D, D5. Spectra are simplified after iCIEF separation and distribution of different drug payload can be observed.

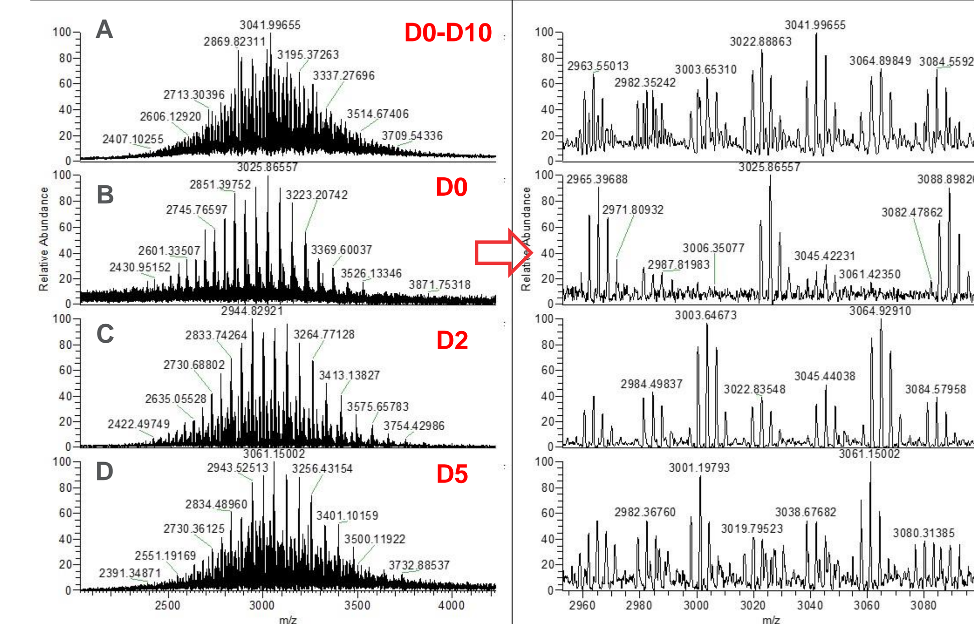


Figure 6. iCIEF- MS spectra and deconvolution results of T-DM1, D0 to D10 (simulation of no iCIEF separation condition). MS signals from adjacent charge states and drug payloads are overlapped, increasing the complexity of the spectra.

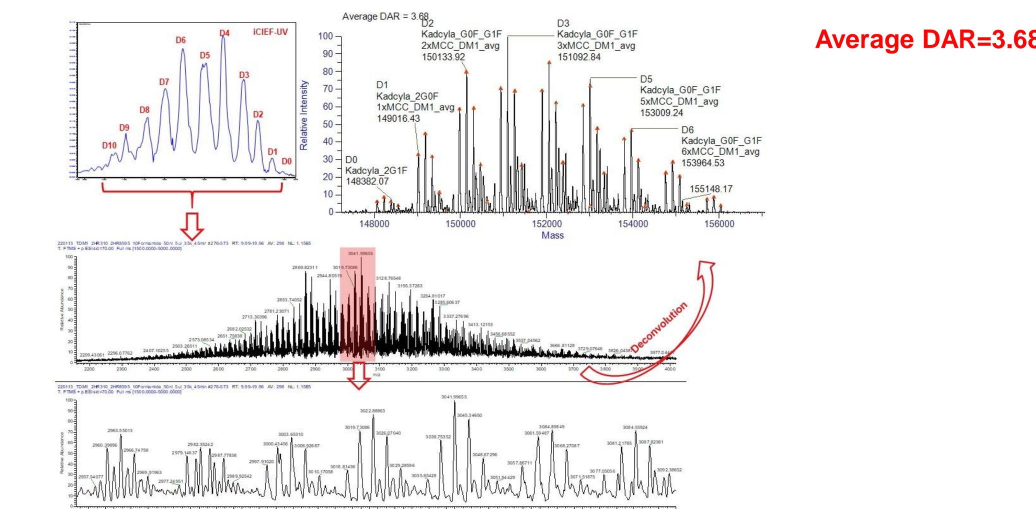


Figure 7. iCIEF- MS spectra and deconvolution results of T-DM1, D0. The complexity of MS spectra was significantly reduced due to iCIEF separation before MS detection.

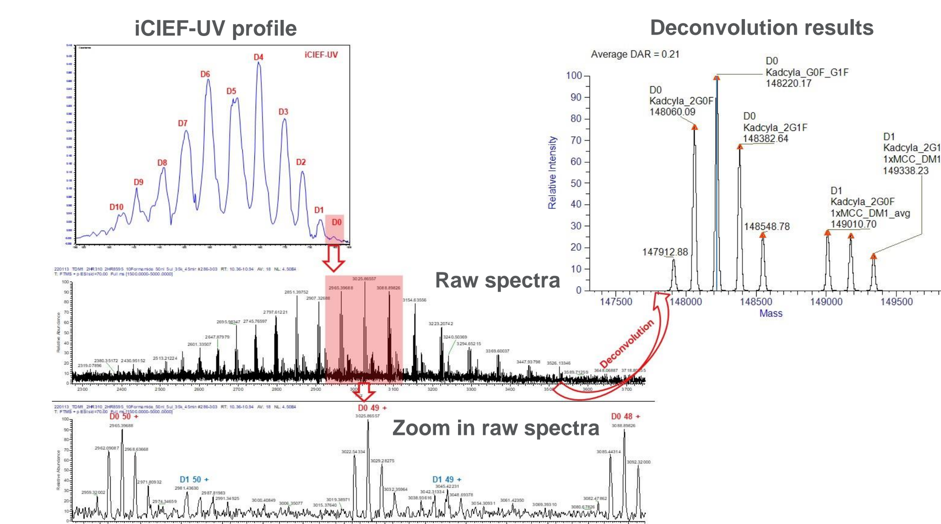


Figure 8. iCIEF- MS spectra and deconvolution results of T-DM1, D2. The complexity of MS spectra was significantly reduced due to iCIEF separation before MS detection.

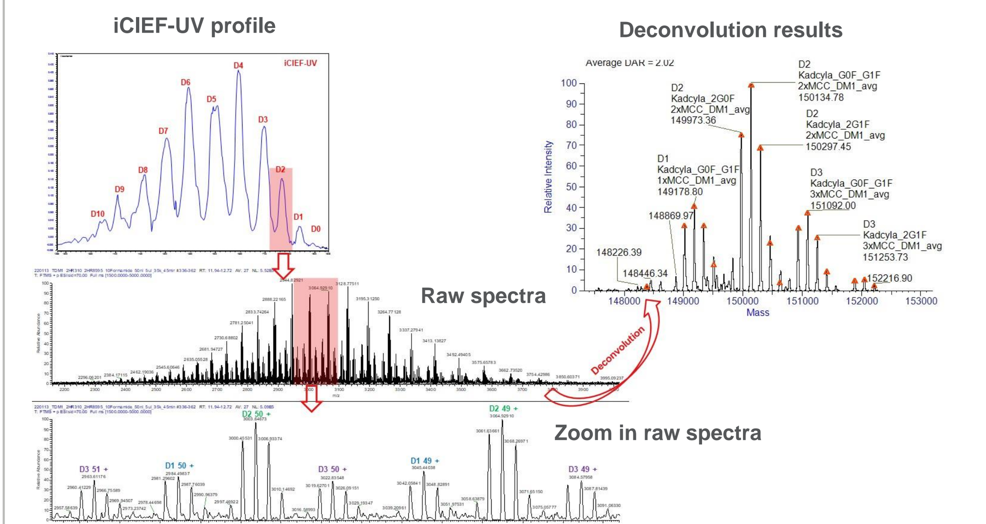
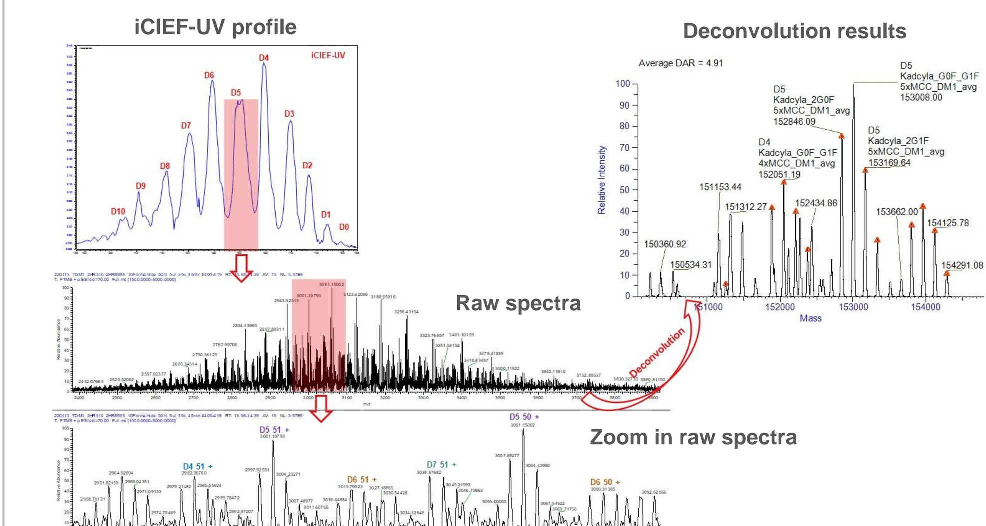


Figure 9. iCIEF- MS spectra and deconvolution results of T-DM1, D5. The complexity of MS spectra was significantly reduced due to iCIEF separation before MS detection.



CONCLUSIONS

- Established and validated the direct iCIEF-MS online coupling workflow for mAb and ADC charge variants separation and intact mass analysis without extra interface.
- Successfully separated and achieved real-time intact protein analysis of pembrolizumab charge variants.
- Successfully separated and achieved real-time intact protein analysis of T-DM1 different drug payload forms.

REFERENCES

1. Zhang X, Zhou Z, Sang M, Huang T, Chen T, Bo T and Du M. In Depth Characterization of mAb Charge Variants with iCIEF Fractionation Followed by Peptide Mapping Analysis. https://assets.thermofisher.cn/TFS-Assets/CMD/posters/sub163-PO66105-ASMS2021-iCIEF-MS-Xiaoxi-v2-DFbrand-cbk-final.pdf

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