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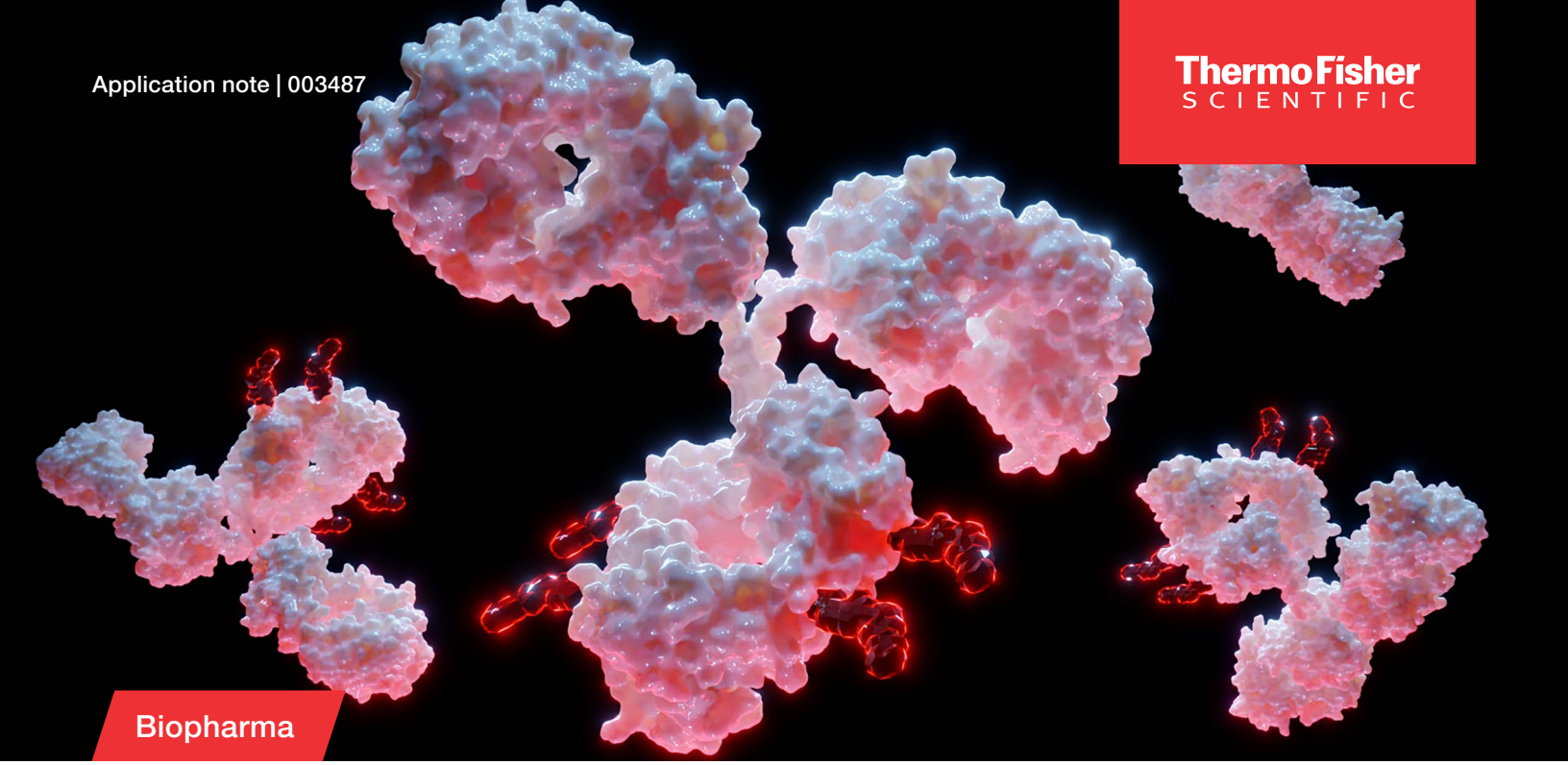

Biopharma

Middle-down analysis utilizing proton transfer charge reduction for accurate localization of payload conjugation sites in an antibody-drug conjugate

Authors

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Keywords

Orbitrap Ascend BioPharma Tribrid mass spectrometer, proton transfer charge reduction (PTCR), electron transfer/higher-energy collisional dissociation (EThcD), middle-down antibody-drug conjugate (ADC)

Application benefits

- Unambiguous localization of payload-conjugation sites
- Increased sequence coverage and elevated number of complementary ion pairs compared to standard MS^2 workflows
- The use of proton transfer charge reduction for subunit analysis of antibody-drug conjugates results in product ion spectra with reduced complexity, enabling spectral matching with higher confidence

Goals

- Demonstrate the capabilities of the Thermo Scientific™ Orbitrap™ Ascend BioPharma Tribrid™ mass spectrometer for extensive characterization of antibody-drug conjugates (ADC) using middle-down mass spectrometry.
- Achieve increased sequence coverage to pinpoint the exact payload-conjugation sites on a variable drug-to-antibody ratio ADC with the aid of proton transfer charge reduction (PTCR).
- Demonstrate the level of spectral decongestion achieved through PTCR and its importance in facilitating reliable product ion matching.

Introduction

Antibody-drug conjugates (ADCs) are emerging as the "magic bullets" in the fight against cancer, owing to their ability to alleviate the systemic toxicity encountered in standard chemotherapy.[1] Their selectivity can be attributed to the building blocks—monoclonal antibody (mAb), drug molecule (payload), linker—working together in a highly orchestrated fashion to have the cytotoxic drug take effect only on intended cellular targets. ADCs are typically synthesized using the stochastic conjugation of payloads to lysine or cysteine residues,[2] which results in a mixture of ADC products having variability in drug-to-antibody ratios (DAR) (i.e., different numbers of payloads attached) also including the precise location of conjugation sites for a given DAR. This heterogeneity has implications for overall product safety and efficacy, since the various DAR forms and positional isomers can show altered stability and pharmacokinetics. Therefore, establishing analytical workflows that enable the reliable and in-depth characterization of these modalities—with respect to both mAb- and ADC-specific attributes—is pivotal for quality control.

For historical reasons, bottom-up mass spectrometry (BU MS) can be considered the default approach for analyzing mAbs and related modalities. In BU MS, the protein is subjected to proteolysis (e.g., by trypsin), and the resulting peptide mixture is analyzed typically by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although the BU workflow does an excellent job at delivering high/full sequence coverage of amino acid sequences, this comes at the cost of (i) introducing artifactual modifications during proteolysis, which distorts the true image of the protein, and (ii) losing information on combinatorial payload locations. On the contrary, top-down (TD) MS involves no enzymatic digestion, with proteins being analyzed in their intact state, thus avoiding associated BU limitations. However, the TD MS analysis of ≈150 kDa species yields limited sequence coverage (~30-40%).[3] Recently, middle-down (MD) MS has taken on a leading role in the structural analysis of mAbs[4] and ADCs[5], owing to its ability to strike a fine balance between the benefits and shortcomings of BU and TD approaches. MD MS relies on mAb digestion with the IdeS protease, followed by reduction of disulfide bonds, resulting in large antibody subunits whose mass (≈25 kDa) comply better with the current state of MS technology—elevating sequence coverages up to 90% when the results of multiple ion activation techniques are combined.[3]

Despite the obvious advantages offered by MD MS, there is still room for improvement regarding the characterization of both standard mAb critical quality attributes (CQA) and ADC-specific features. The additional layer of challenges imposed by drug-conjugation necessitates the use of highly sophisticated analytical platforms that allow (i) the separation of different DAR species and (ii) sequencing of the separated subunits with respect to identifying payload conjugation sites. Localization of drug-conjugation has proven to be an exceptionally hard task, especially when variable-DAR ADCs are under investigation. Although ion activation techniques commonly used in such workflows—namely higher energy collisional dissociation (HCD), electron-transfer dissociation (ETD) either as a standalone method or combined with HCD post-activation (termed EThcD), and ultraviolet photodissociation (UVPD)—demonstrate outstanding performance in generating potentially valuable fragments, product ion spectra are generally heavily congested. Consequently, the interpretation of such spectra remains complicated.

Simplification of product ion spectra can be carried out by dispersing fragment ion populations over a wider *m/z* range, by a process termed proton transfer charge reduction (PTCR). PTCR subsequent to EThcD has proven to be exceptionally beneficial for MD MS workflows; sequence coverage exceeding 85% could be obtained for antibody subunits from a single run.[6] EThcD generally yields more informative fragment ion mass spectra and hence higher sequence coverage than ETD alone due to its ability to separate the non-dissociative electron transfer (ETnoD) products that are held together by intramolecular non-covalent interactions. Hereinafter, we demonstrate the efficacy of EThcD combined with PTCR in localizing payload conjugation sites on a variable-DAR ADC mimic.[7]

Herein, we highlight the importance of spectral simplification by PTCR for the characterization of a structurally more complex class of biotherapeutics using an Orbitrap Ascend BioPharma MS. Subunits of ≈25 kDa (namely: Lc, Fd', and Fc/2) were obtained from the IdeS digestion and disulfide bond reduction of a variable-DAR ADC mimic. These subunits were chromatographically separated and subjected to EThcD MS^2 with and without PTCR MS^3 (Figure 1A). Charge reduction of product ions is achieved by transferring a proton to the PTCR reagent anion (Figure 1B). The workflow enabled the unambiguous localization of conjugation sites on the Fd' subunit—the trickiest.

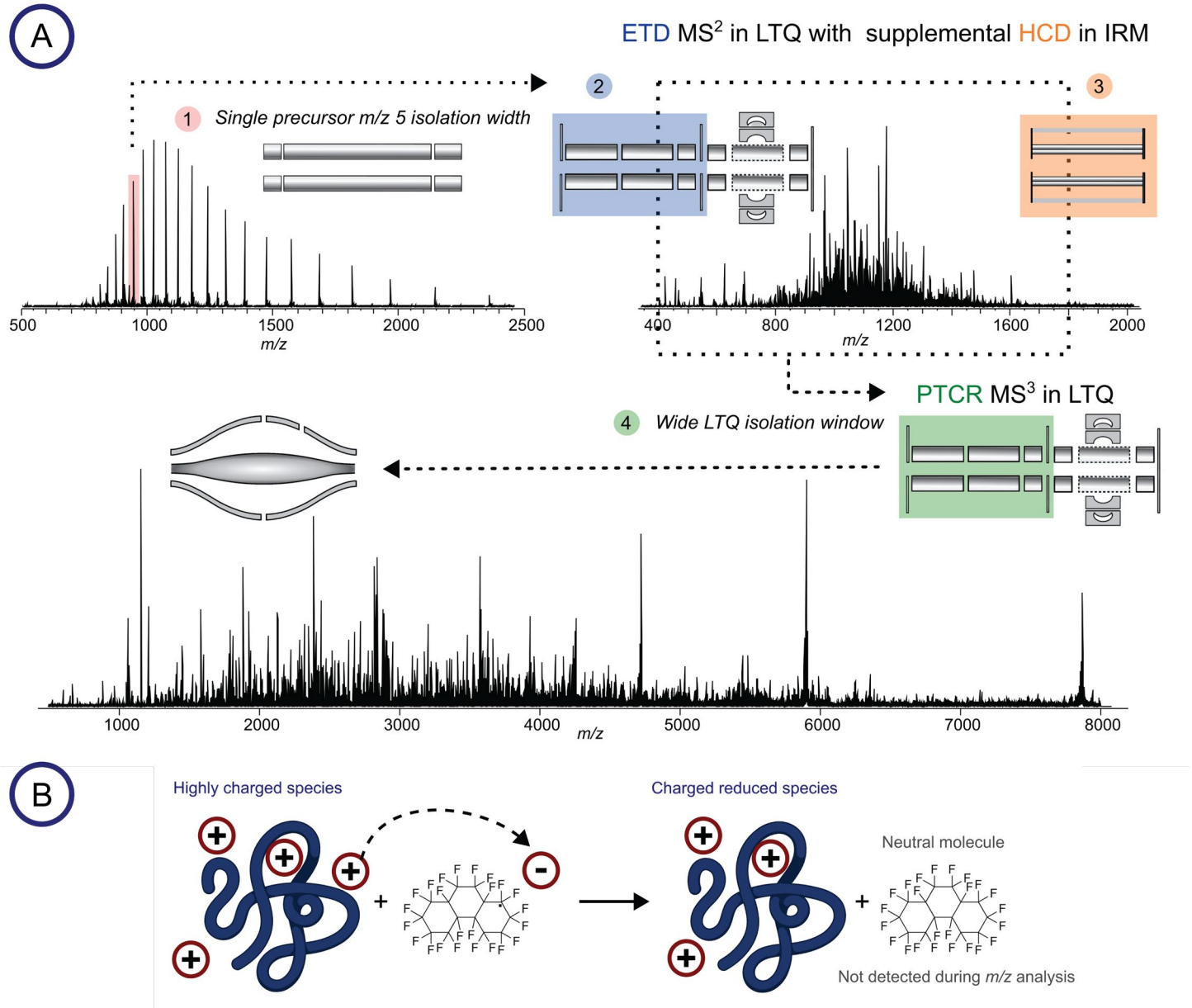

Figure 1. Mass spectrometry analysis carried out using an Orbitrap Ascend BioPharma MS. (A) Main steps of data acquisition, namely: (1) quadrupole isolation of an individual precursor charge state, (2) ETD reaction performed in the high-pressure cell of the dual-pressure LTQ followed by (3) the HCD-based supplemental activation of ETD products in the back ion-routing multipole (IRM), and—if experiments are performed at the MS^3 level—(4) isolation of EThcD products, that are subjected to PTCR prior to detection in the Orbitrap mass analyzer. (B) Reaction scheme of the PTCR event: a single proton is transferred from a multiply charged cation to the PTCR reagent anion, yielding a charge-reduced cation and a neutral molecule.

Experimental

Sample preparation

SILu™ SigmaMAb Antibody Drug Conjugate (ADC) mimic (MSQC8, MilliporeSigma) was used for all experiments. Sample preparation consisted of a digestion step with IdeS enzyme (Genovis) following the manufacturer's protocol and a reduction step (45 min, 55 °C) using a mixture of 100 mM tris (2-carboxyethyl) phosphine (TCEP) and 100 mM dithiothreitol (DTT) in 6.5 M guanidinium chloride, yielding three ≈25 kDa subunits (Fc/2, Lc, and Fd) with a varying number of attached payloads. Sample clean-up was performed using Thermo Scientific™ Zeba™ spin desalting columns with a molecular weight cutoff of 7,000, equilibrated in 0.1% formic acid.

Reagents and consumables

- Fisher Scientific™ Water, Optima™ LC/MS grade, (P/N W81)
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A955-1)
- Fisher Scientific™ Formic acid, Optima™ LC/MS grade (P/N A11710X1AMP)
- Thermo Scientific™ 8 M Guanidine-HCl Solution (P/N 24115)
- Thermo Scientific™ Pierce™ TCEP-HCl (P/N 20490)
- Thermo Scientific™ Pierce™ DTT (P/N 20290)
- Thermo Scientific™ Zeba™ Spin Desalting Columns, 7K MWCO, 0.5 mL (P/N 89883)
- FabRICATOR™ (IdeS enzyme, Genovis)
- Thermo Scientific™ Autosampler Vial Screw Thread Caps (P/N C5000-54B)

Chromatography

Reversed-phase LC separations were carried out using a Thermo Scientific™ Vanquish™ Flex UHPLC system equipped with:

- Thermo Scientific™ System Base Vanquish Flex/Horizon (P/N VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A-02)
- Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
- Thermo Scientific™ Viper™ MS Connection Kit for Vanquish™ LC systems (P/N 6720.0405)
- Thermo Scientific™ MAbPac™ RP column, 150 mm × 1 mm, 4 µm particle size (P/N 303184)

Table 1. Overview of LC conditions

LC conditions	
Column	MAbPac RP, 150 mm × 1 mm, 4 µm
Mobile phase A	Water with 0.1% formic acid (v/v)
Mobile phase B	ACN with 0.1% formic acid (v/v)
Flow rate	120 µL/min
Column temp.	80 °C
Injection	1 µg
Gradient	Time (min) % B 0 5 0.5 5 1 22 6.5 29 13 36 14.5 90 16.5 90 17 5 20 5

Mass spectrometry

All LC-MS^n experiments were carried out on an Orbitrap Ascend BioPharma MS equipped with the ETD, UVPD, PTCR, and High Mass Range MS^n+ (HMR^n+) options. Thermo Scientific™ Xcalibur™ software v. 4.2 SP1 and Orbitrap Tune v. 4.0 instrument control software were used. Orbitrap mass spectra were recorded without automatic noise removal, referred to as full profile mode. MS parameters used in this study are provided in Table 2.

Data analysis

MS^2 and MS^3 mass spectra were averaged and exported as single spectrum .raw files using Thermo Scientific™ Freestyle™ 1.8 software. Thermo Scientific™ BioPharma Finder™ 5.2 software was used for generating the neutral fragment mass via the Xtract algorithm and for fragment matching applying Top-Down default deconvolution method. The following fragment ion types were considered depending on the different fragmentation types applied: HCD: *b/y;* EThcD: *c/z* and *b/y;* UVPD: *a, a+, b, c, x, x+, y, y–, z·* with "+" and "-" indicating addition or loss of a hydrogen, respectively. The main parameters for the Xtract algorithm varied depending on the file type and were modified from the default method. The main parameters for the Xtract algorithm in the case of MS^2 spectra were as follows: mass tolerance 10 ppm; signal-to-noise (S/N) 7; fit factor 75%, remainder threshold 25%. For PTCR MS^3 files, the S/N was set to 5, fit factor to 70%, and remainder threshold to 25%.

Table 2. Overview of MS settings

MS source settings			
Spray voltage (V)	+3,400		
Sheath gas (a.u.)	35		
Aux gas (a.u.)	10		
Sweep gas (a.u.)	0		
Ion transfer tube temp. (°C)	320		
Vaporizer temp. (°C)	150		
Source fragmentation (V)	15		
Application mode	Intact Protein		
Pressure mode	Low (1 mTorr)		
Acquisition			
Settings	**MS¹**	**Targeted MS²**	**Targeted MS³**
Resolution (at 200 *m/z*)	240,000	240,000	240,000
Scan range (*m/z*)	500-4,000	350-2,000	500-8,000
RF Lens (%)	60	60	60
AGC target value (%)	1,000	2,000	2,000
Max injection time (ms)	507	507	507
Microscans	2	2	2
ETD quadrupole isolation window (*m/z*)	-	5	5
ETD reagent AGC target value	-	7.00E+05	7.00E+05
ETD reagent - max inject time (ms)	-	200	200
ETD reaction time (ms)	-	5	4
Supplemental activation coll. energy (%)	-	20	20
PTCR ion trap isolation window (*m/z*)	-	-	1,800
PTCR reagent AGC target value	-	-	2.00E+06
PTCR reaction time (ms)	-	-	40

Results and discussion

Data acquisition was carried out following a classical targeted approach: a preliminary LC-MS¹ analysis was performed to define the targets and their retention times for the subsequent LC-MS² run. The accurate intact masses obtained from MS¹ analysis enabled the putative assignment of peaks, where mass shifts of 668 Da were indicative of payload attachment(s) to the Lc and Fd' subunits. In addition to the separation of subunits bearing an increasing number of payloads, the (Fd'+1 drug) and (Fd'+2 drugs) species presented two well-resolved chromatographic peaks (Figure 2). These isobaric species signify the presence of positional isomers (hereinafter referred to as Fd'+1a, Fd'+1b and Fd'+2a, Fd'+2b). Such conjugation-site heterogeneity is to be expected from variable-DAR ADCs. The conjugation strategy relies on the partial reduction of the antibody, potentially cleaving three disulfide bonds—two interchain disulfides between the heavy chains (Hc) and one linking the Hc to the Lc. The reactive thiols formed upon reduction of intermolecular disulfide bonds serve as points of attachment for the drug molecule, resulting in antibody scaffold accommodating up to three payloads on the Hc (more precisely, the Fd') in various arrangements and one payload on the Lc. The average DAR was derived as 4.02 (value reported by manufacturer: 4 ± 0.8), based on the calculation determining the area under the curve (AUC) ratio of payload-containing and overall subunit populations separately for Lc and Fd'-related species, using the formula below:[5]

$$DAR = 2 \times \left(\frac{AUC_{Lc+1}}{AUC_{Lc+0} + AUC_{Lc+1}} + \frac{AUC_{Fd'+1} + 2AUC_{Fd'+2} + 3AUC_{Fd'+3}}{AUC_{Fd'+0} + AUC_{Fd'+1} + AUC_{Fd'+2} + AUC_{Fd'+3}}\right)$$

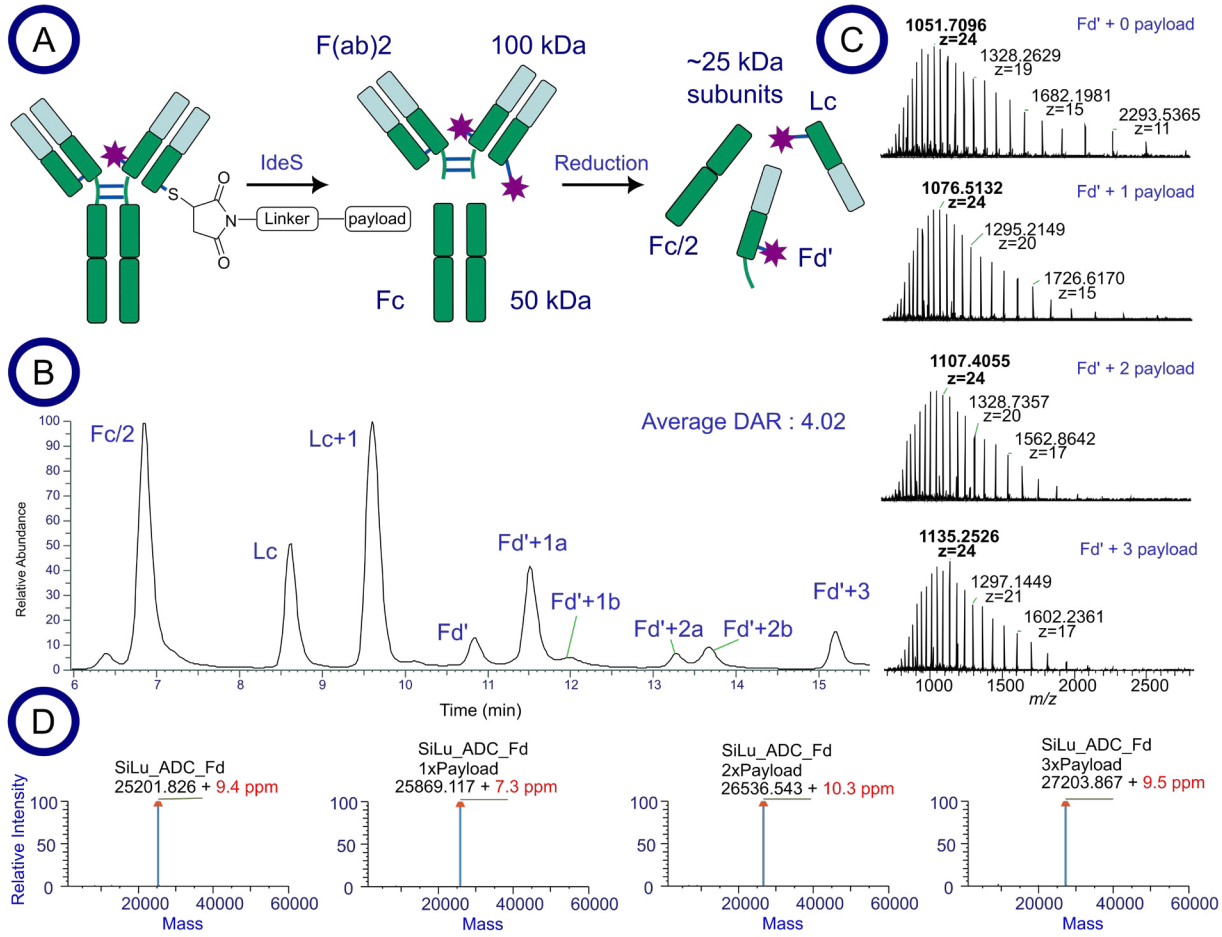

Figure 2. MS¹ subunit analysis of ADC mimic. (A) Sample preparation including IdeS digestion and disulfide bond reduction, generating ≈25 kDa subunits. (B) Base peak chromatogram obtained for the IdeS-digested and reduced ADC mimic. Fd'+1a and Fd'+1b are isobaric species containing 1 payload that differ only in conjugation site (same logic holds true for Fd'+2a and Fd'+2b). (C) MS¹ spectra of selected peaks. The incremental mass shifts caused by the increasing number of payload attachments on the Fd' subunit is highlighted in bold in the spectra (at z=+24). (D) Deconvolution results for Fd' species, indicating the theoretical monoisotopic mass and observed mass error.

A single charge state (z=+29) for each Fd' species was selected and subjected to EThcD using the parameters specified in Table 2. The focus of this study was placed on the extensive characterization of the Fd' subunit and all its varieties, paying particular attention to the precise localization of conjugation sites found on isobaric species. The EThcD MS² analysis provided good sequence coverage (≈50%), and in the case of peaks Fd'+1a and Fd'+1b, diagnostic product ion spectra were obtained that prove the exact position of payload-attachment.

Although the MS² spectra obtained by EThcD are rich in sequence information, certain low S/N fragment ions remain unnoticed due to the heavy overlapping of isotopic distributions. Spectral congestion is inevitable considering the large number of multiply charged product ions generated in MS² events. However, the application of PTCR substantially simplifies such spectra by decreasing the charge state of product ions and hence dispersing the ion population over a wider *m/z* space (Figure 3A). The resulting decluttered spectra afforded a generous increase in both total number of fragments (Figure 3B) and the number of complementary ion-pairs identified (Figure 3C), the latter being essential for increasing the confidence in assigning backbone-cleavage products. Although PTCR MS³ generally enhances sequence coverage substantially, there is a low proportion of product ions unique to EThcD MS². This can be attributed to relatively small fragment ions (low charge, low mass) being neutralized during the PTCR reactions and hence going undetected.

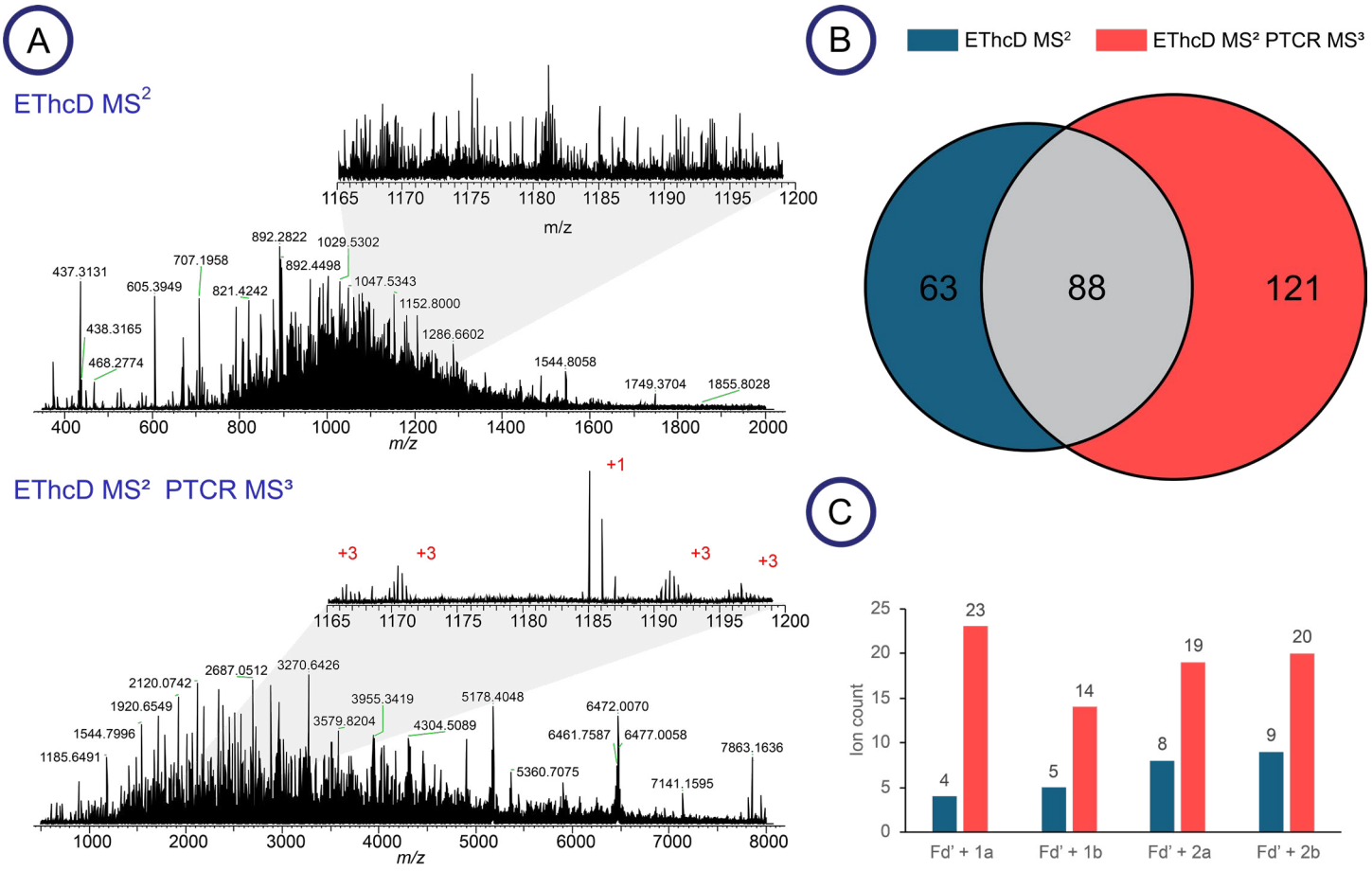

Figure 3. Effect of PTCR. (A) Comparison of MS^2 and MS^3 spectra of the Fd'+1a subunit. Insets (displaying the *m/z* 1165–1200 portion of the spectra) emphasize the utility of PTCR in spectral decongestion. (B) Total number of fragments identified for the Fd'+1a subunit with and without PTCR. (C) Bar graph illustrating the increase in the number of complementary ion pairs due to PTCR.

Beyond the general improvement in sequence coverage upon the involvement of PTCR, the MS^3 workflow provided the means to eliminate the element of ambiguity from localizing payload-conjugation sites on both Fd'+2 species. Considering that the cysteine residues potentially carrying the payloads are all localized towards the C-terminal and in proximity to each other (C224, C230, and C233), the extensive sequencing of this region is of the highest importance. Although diagnostic product ions from this region are most likely generated in a simple MS^2 experiment, their signals are not matched due to ion signal overlap. With the use of PTCR it was possible to uncover these diagnostic ions (Figure 4).

While the EThcD MS^2 – PTCR MS^3 workflow presented here offers the possibility to localize payload conjugation sites with no ambiguity (Table 3), the utilization of multiple ion activation methods available on the Orbitrap Ascend BioPharma MS can further expand the depth of sequence information. Due to the complementary nature of the ion dissociation methods available on this instrument (precursor activation based on interactions with neutral molecules, electrons, or photons for HCD, ETD, and UVPD, respectively), the integration of datasets can both strengthen the confidence in existing identification as well as provide supplementary pieces of information, ultimately yielding higher sequence coverage (Figure 5, Table 3).

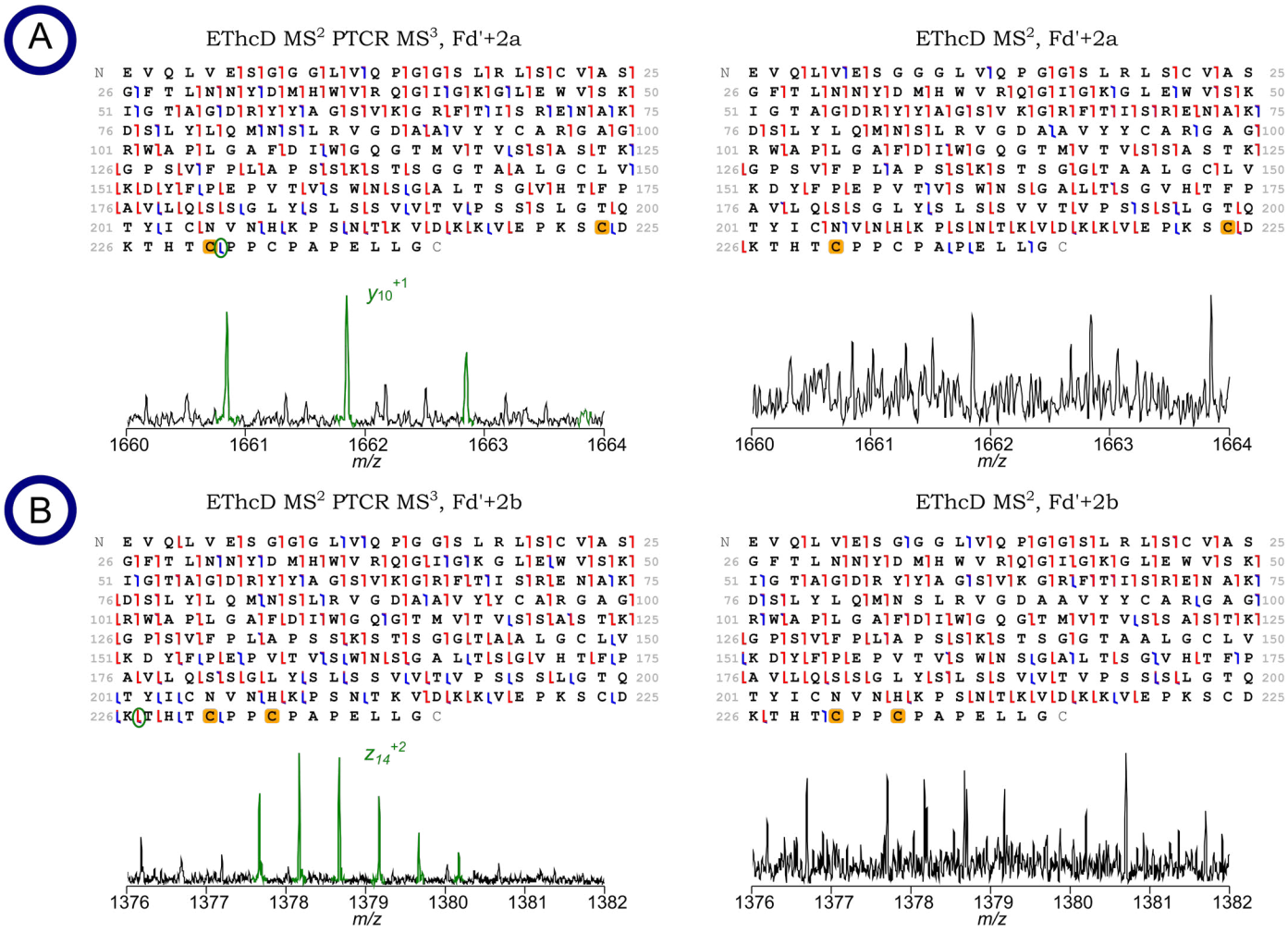

Figure 4. Payload localization with the help of PTCR. Fragmentation maps and product ion spectra for the (A) Fd'+2a and (B) Fd'+2b species. Left panel: results obtained with EthcD MS^2 – PTCR MS^3. Spectra show the diagnostic ions revealed with the use of PTCR (circled in green on the fragmentation maps). Right panel: results obtained with EthcD MS^2. Diagnostic product ions y_{10}^{1+} and z_{14}^{2+} are not apparent in the corresponding section of the MS^2 spectra. Highlighted in orange are the cysteine residues carrying the payload.

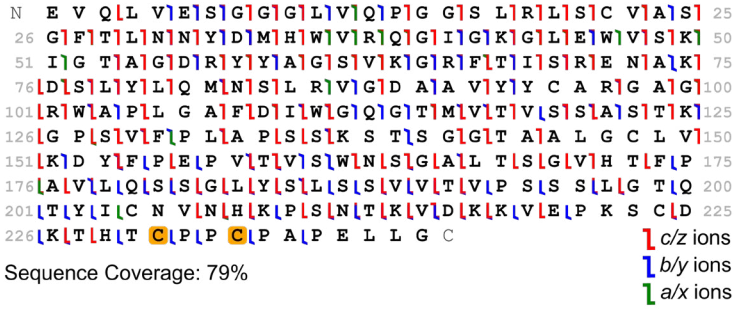

Figure 5. Fragmentation map of the Fd'+2b subunit obtained by combining results of targeted PTCR methods utilizing HCD, ETD, EThcD, and UVPD as the MS^2 event. Blue, red, and green lines indicate *b-y, c-z*, and *a-x* fragment ion types, respectively.

Subunit		Fd' + 1a		Fd' + 1b		Fd' + 2a		Fd' + 2b	
Experiment		Sequence Coverage (%)	Localization	Sequence Coverage (%)	Localization	Sequence Coverage (%)	Localization	Sequence Coverage (%)	Localization
MS^2	HCD	26	Ambiguous	27	Ambiguous	26	Ambiguous	25	Ambiguous
	ETD	46	C224	40	Ambiguous	42	Ambiguous	41	Ambiguous
	UVPD	35	Ambiguous	32	Ambiguous	28	Ambiguous	27	Ambiguous
	EThcD	52	C224	49	C230	48	Ambiguous	49	Ambiguous
PTCR - MS^3	HCD	22	C224	27	Ambiguous	24	Ambiguous	25	Ambiguous
	ETD	55	C224	49	Ambiguous	24	Ambiguous	50	C230+C233
	UVPD	42	Ambiguous	41	Ambiguous	39	Ambiguous	39	C230+C233
	EThcD	61	C224	57	C230	55	C224+C230	60	C230+C233
	Combined	79	C224	82	C230	79	C224+C230	79	C230+C233

Table 3. Results for MS^2 and PTCR-MS^3 experiments using multiple ion activation techniques for Fd' subunits containing payloads.

Conclusions

- Here, we present the exceptional performance of the Thermo Scientific Orbitrap Ascend BioPharma Tribrid mass spectrometer in the comprehensive characterization of the payload conjugation sites of a variable-DAR antibody-drug conjugate.
- Spectral decongestion via PTCR resulted in a 16–20% increase in sequence coverage obtained for subunits containing varying numbers of drug molecules.
- The targeted PTCR-MS^3 method enabled the unambiguous localization of payload conjugation on a variable-DAR ADC by relying on a single ion activation technique (EThcD).
- Sequence coverage could be further enhanced (>75%) by combining the results of complementary ion activation methods. The availability of multiple ion activation techniques is invaluable for localizing co-occurring modifications (e.g. oxidation, deamidation), providing deeper insights into the more delicate heterogeneities/microvariabilities furnishing the mAb scaffold.

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