APPLICATION NOTE 74160

Proton transfer charge reduction (PTCR)

improves spectral matching and sequence coverage in middle-down analysis of monoclonal antibodies

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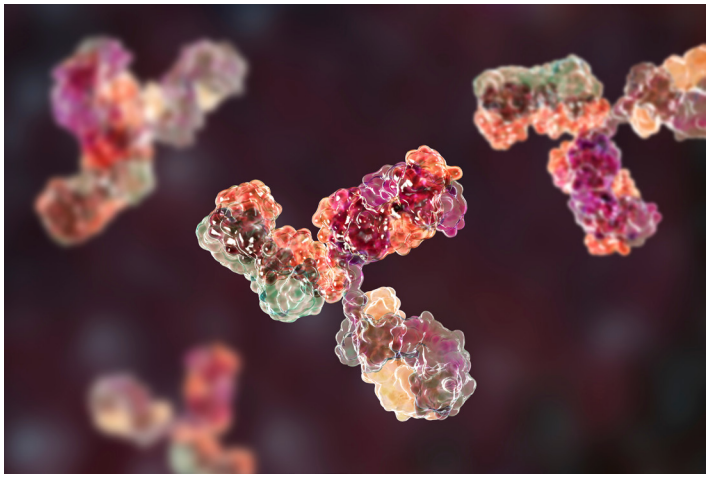


Application benefits

- Improved sequence coverage for 25 kDa subunits of monoclonal antibodies generated by rapid IdeS proteolysis compared to standard MS^2-based methods.
- Exceptional reduction in spectral congestion via application of proton transfer charge reduction, leading to increased confidence in product ion matching.
- Unparalleled capability of identification of large product ions complementary to shorter N- and C-terminal fragments that boosts the confidence in localization of any potential post-translational modification on antibodies.

Goal

- Compare the performance offered by standard electron transfer dissociation tandem mass spectrometry (ETD MS^2) and ETD MS^2 followed by proton transfer charge reduction (PTCR MS^3) on a Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer.
- Elucidate the amino acid sequence of a standard antibody (immunoglobulin G1) by middle-down mass spectrometry.
- Demonstrate the potential of PTCR in simplifying fragment ion spectra in conjunction with the high *m/z* range achievable in the Orbitrap mass analyzer by applying the HMR^n option (up to *m/z* 8000), which overall facilitates product ion matching.
- Provide optimal experimental conditions to characterize any monoclonal antibody or antibody-drug conjugate using multiple ion activation techniques in combination with PTCR.

Introduction

Recombinant monoclonal antibodies (mAbs) are currently the most important class of biotherapeutic molecules. Due to their ability to treat a variety of conditions, several mAbs rapidly became blockbuster drugs dominating the biopharmaceutical market, while many more mAb-based drugs are currently undergoing clinical trials and await approval. Additionally, several other biologics such as antibody-drug conjugates (ADC) based on mAb scaffolds are now available on the biopharmaceutical market. For developers of these biotherapeutics, characterization of the primary sequence of these biologics is critical. Optimal utilization of the most powerful analytical tools and methods available to robustly achieve this is essential.

Middle-down mass spectrometry (MD MS) has become a widely adopted standard approach in the structural analysis of mAbs.[1] Its success derives from the fact that MD MS leverages key benefits from bottom-up mass spectrometry (BU MS) and top-down mass spectrometry (TD MS). BU MS relies on the in-solution digestion of mAbs by proteomics-grade proteases (e.g., trypsin, Glu-C, Lys-C) followed by liquid chromatography-tandem mass spectrometry (LC-MS^2) analysis of the resulting short proteolytic peptides. Although BU MS can lead to complete sequence coverage of a mAb, the lengthy sample preparation can introduce unwanted artifacts, such as oxidation of methionine or deamidation of asparagine and glutamine residues. Presence of the procedure-introduced modifications would in turn prevent the possibility to identify and quantify the endogenous modifications present on mAbs. In contrast, TD MS eliminates most sample preparation steps and enables direct analysis (typically via LC-MS^2) of intact 150 kDa mAbs retaining their original quaternary structure. However, the sequence coverage achievable by TD MS is limited typically to 30–40% by the presence of inter- and intra-molecular disulfide bonds; additionally, the data analysis is complicated by the high peak density, which results in a very high incidence of overlapping isotopic *m/z* peak envelopes associated with the numerous fragment ion species.[2]

While both BU and MD rely on proteolysis for mAb characterization, MD uses the IdeS protease that cleaves mAbs at a single site, with good efficiency in a short amount of time. By following IdeS digestion with disulfide bond reduction and subunit denaturation, the sample preparation procedure generates three ~25 kDa subunits, namely Lc, Fd', and Fc/2, that can be easily separated by reversed-phase liquid chromatography using Thermo Scientific™ MAbPac™ columns (Figure 1A). Using Thermo Scientific Tribrid Orbitrap mass spectrometers, the sequence coverage for each of the three ~25 kDa subunits analyzed by LC-MS^2 is typically >60% when multiple ion activation methods are applied through multiple LC-MS^2 experiments, and the resulting fragmentation maps are combined.[2,3] Nonetheless, MD MS has also limitations. Specifically, the larger subunit, Fd', is often not sufficiently sequenced, with one or more complementarity determining regions (CDRs) remaining uncharacterized. Furthermore, due to the complexity of MS^2 spectra of 25 kDa subunits, only the most intense product ions are typically matched; these are often the smaller fragment ions, whose larger complementary product ions are rarely identified.

Here we describe the advantages offered by proton transfer charge reduction (PTCR) for MD MS. Available as an option for the Orbitrap Eclipse Tribrid MS together with electron transfer dissociation (ETD), PTCR subsequent to ETD reduces spectral congestion in the product ion spectra of mAb subunits, leading to improved sequence coverage as well enabling identification of many large complementary product ions that strengthen the confidence in both the assigned sequence and the correct localization of post-translational modifications (PTMs). Data analysis performed using TDValidator (Proteinaceous, Inc.) enables the possibility of validating product ion matching and removing false positives (i.e., incorrect assignments).

Experimental

Sample preparation

The commercially available mAb SILu™Lite SigmaMAb Universal Antibody Standard (MilliporeSigma) was used for all experiments presented in this application note. For subunit analysis, the sample was digested with IdeS enzyme (Genovis) according to the manufacturer's protocol, followed by a reduction step by incubation with 50 mM tris(2-carboxyethyl)phosphone (TCEP) at 55 °C in 8 M guanidine hydrochloride for 45 min to yield Lc, Fc/2, and Fd' subunits (Figure 1A). Reduced subunits were desalted using a Thermo Scientific ™ Zeba™ spin column equilibrated in 0.1% formic acid (v/v) prior to LC-MS analysis.

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™ Bond-Breaker™ TCEP Solution, Neutral pH (P/N 77720)
- 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 chromatography was carried out on a Thermo Scientific™ Vanquish™ Horizon UHPLC system, consisting of:

- 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)
- MS Connection Kit Vanquish (P/N 6720.0405)

For all mAb subunit analyses, 1 µg of digested and reduced mAb was loaded onto a 1.0 × 150 mm Thermo Scientific™ MAbPac™ RP column with 4 µm particle size (P/N 303184) and separated with a non-linear solvent gradient (details in Table 1).

Table 1. Overview of LC conditions: columns, solvents, flow rates, column temperature, and gradient applied for MS analysis of mAb subunits under denaturing conditions

LC conditions		
Column	MabPac RP, 4 µm, 1.0 × 150 mm	
Mobile phase A	Water with 0.1% formic acid (v/v)	
Mobile phase B	Acetonitrile with 0.1% formic acid (v/v)	
Flow rate	120 µL/min	
Column temperature	70 °C	
Gradient:	Non-linear	
	Time (min)	**% B**
	0	5
	2.5	27
	7	29
	10	35
	14	85
	16	85
	16.5	5
	20	5

Mass spectrometry

All MS experiments were performed on an Orbitrap Eclipse mass spectrometer equipped with the ETD and PTCR (includes reagent/internal calibrant source, reagent inlets, and ion-ion enabling electronics) and High Mass Range MS^n (HMR^n) mode options. The instrument was operated with Thermo Scientific™ Xcalibur™ 4.2 SP1 software and controlled by Orbitrap Eclipse 3.4 instrument control software. Orbitrap mass spectra were recorded without automatic noise removal (i.e., in "full profile" mode, accessible through the Diagnostic menu of the instrument control software). All MS parameters used in this study are summarized in Table 2. The MS^3 PTCR experiments significantly reduce the total amount of charge of the analyzed product ions in comparison to the amount charge of MS^2 precursor population originally injected. Therefore, the total ion current (TIC) observed in the MS^3 spectrum measured in the Orbitrap analyzer was used as a "space charge" correction term for the frequency to *m/z* conversion calculation preformed on a scan per scan basis to provide improved *m/z* accuracy. This type of correction is enabled as a diagnostic patch available upon request.

Table 2. MS parameter settings for all experiments

MS conditions	Intact subunit (Reduced mAb)	Targeted MS^2	Targeted MS^2 & MS^3
Method type	Full MS	tMS^2	tMS^2-tMS^3
Runtime (min)	25	25	25
Scan range (*m/z*)	500–2,000	400–2,000	500–8,000
Resolution at (*m/z* 200)	120,000	120,000	120,000
RF Lens (%)	30	30	30
ETD quadrupole Isolation window (*m/z*)	—	3	3
PTCR ion trap Isolation window (*m/z*)	—	—	1,500
Cation AGC target value (%)	100	2,000	2,000
Max inject time – cation (ms)	200	500	500
ETD reagent AGC target value	—	1.00E+06	1.00E+06
Max inject time – ETD reagent (ms)	—	50	50
PTCR reagent AGC target value	—	—	2.00E+06
Max inject time – PTCR reagent (ms)	—	—	50
ETD reaction duration (ms)	—	3	2
PTCR reaction duration (ms)	—	—	20
Microscans	3	3	3
MS source setting			
Spray voltage (+)*	3,800		
Capillary temp. (ºC)	320		
Sheath gas (a.u.)*	25		
Aux gas (a.u.)	5		
Sweep gas (a.u.)	0		
Vaporizer temperature (ºC)	150		
Source fragmentation (V)*	15		
Application mode	Intact Protein		
Pressure mode	Low (3 mTorr)		
Acquisition mode	Full Profile		

*requires optimization

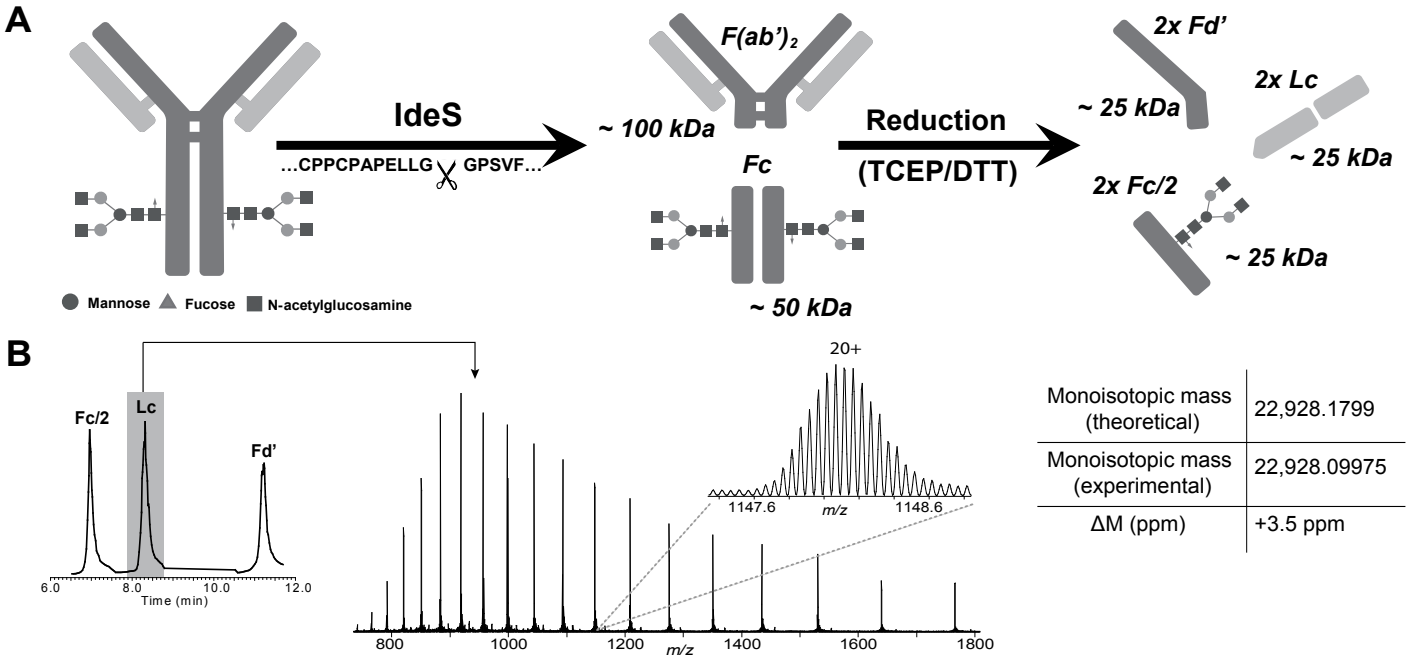
Data analysis

Acquired MS^2 and MS^3 fragmentation spectra were averaged and exported as single spectrum .raw files using the Thermo Scientific™ Freestyle™ 1.4 software. Thermo Scientific™ BioPharma Finder™ 4.1 software was employed to generate the neutral fragment mass via Xtract and to provide fragment matching, assignment sequence coverage maps from these .raw files using Top-Down Default deconvolution method. The main parameters for Xtract varied depending on the file type and modified from the default method. For ETD MS^2 spectra, the following parameters were applied: mass tolerance 10 ppm; signal-to-noise (S/N) 7; fit factor 75%, remainder threshold 25%. For ETD MS^2 – PTCR MS^3 files, the S/N was set to 5, fit factor to 70%, and remainder threshold to 25%. These parameters were selected based on the results obtained by manually validating one MS^2 and one MS^3 file using TDValidator (Proteinaceous, Inc.); the Xtract parameters reported here lead to fragmentation maps highly similar to the manually curated ones.

Results and discussion

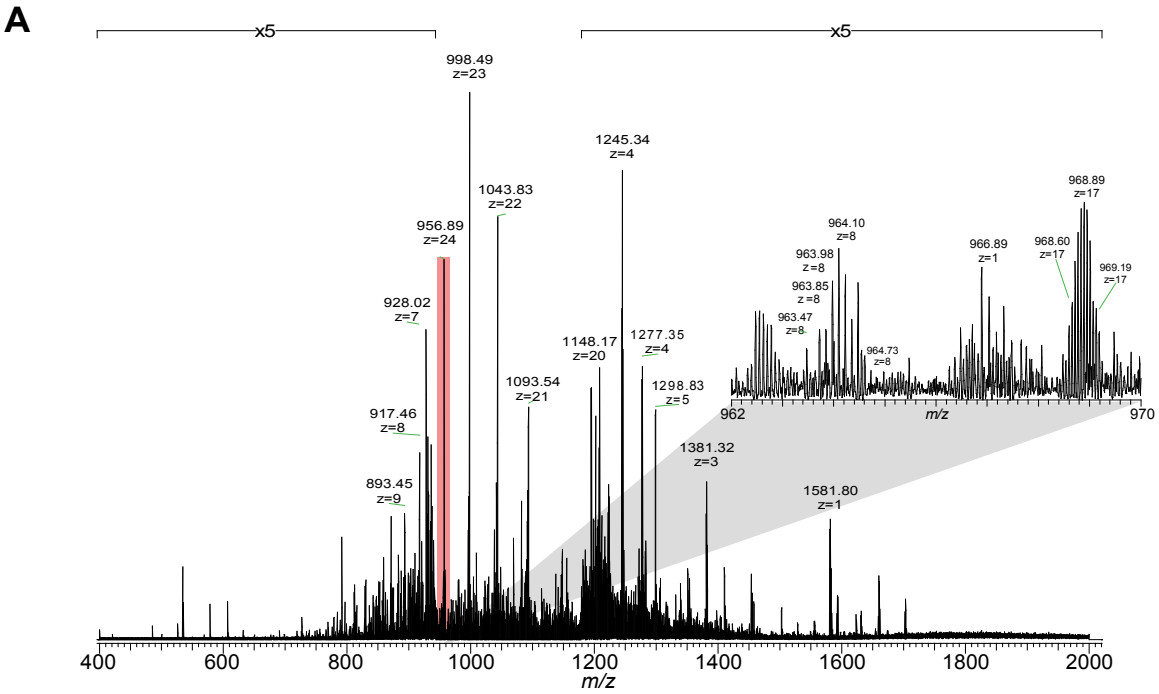
The data presented in this work are based on a traditional targeted data acquisition strategy, to analyze a single precursor *m/z* per subunit. Spectral S/N was improved by averaging multiple fragmentation spectra collected in a single LC run. In practical terms, the standard MS^2 workflow can be summarized as follows: (i) an MS^1-only LC-MS run is performed; this allows the analyst to confirm the results of the IdeS digestion and sample preparation (typically, shoulders on the sides of main chromatographic peaks are indicative of incomplete disulfide bond reduction or of the presence of multiple proteoforms of a certain subunit), as well as to monitor spray stability, elution times of the three mAb subunits and charge state distributions obtained by electrospray ionization (Figure 1B). (ii) For each subunit, a single charge state is selected for ETD MS^2 fragmentation, which is performed using the parameters listed in Table 2 above. ETD MS^2 spectra are collected throughout the elution of each subunit, and they can be averaged and exported as a single spectrum .raw file using Freestyle software (Figure 2). (iii) Averaged ETD MS^2 spectra can be analyzed using BioPharma Finder software and graphical fragmentation maps can be generated.

In the case of the Lc subunit of SILuLite mAb, using the LC gradient specified in Table 1, 25 spectra were averaged across the elution profile (for a total of 50 microscans). Matching only *c*- and *z*-type product ions using BioPharma Finder software, the resulting sequence coverage was 46% with mass accuracy of the identified fragments of 2.9 ppm RMS (Figure 2). Notably, despite the identification of 11 product ions ranging from 10 to 15 kDa (e.g., c_{121}, z_{116}), only three complementary *c*/*z*-ion pairs are found in the fragmentation map.



Monoisotopic mass (theoretical)	22,928.1799
Monoisotopic mass (experimental)	22,928.09975
ΔM (ppm)	+3.5 ppm

Figure 1. (A) Schematics of IdeS proteolysis and subsequent reduction and denaturation of digestion products into three 25 kDa subunits; (B) representative TIC chromatogram and MS^1 spectrum of the Lc subunit



B

N Q S A L T⌉Q P R S⌉V⌉S⌉G⌉S P⌉G⌉Q⌉S V T I S C T⌉G⌉T⌉ 25
26 S⌉S D I⌉G G Y⌉N⌉F V S W Y Q Q⌉H P G⌉K A P⌉K⌊L⌉M I 50
51 Y⌉D A⌉T⌉K⌉R P S⌉G V P⌉D⌉R⌉F S⌉G⌉S⌉K⌉S⌉G⌉N⌉T⌉A⌉S⌉L 75
76 T⌉I⌉S⌉G⌉L⌉Q⌉A⌉E D⌉E⌉A D Y Y C C S⌉Y⌉A G D⌉Y⌉T P G 100
101 V V F⌉G⌊G G T⌊K L T V L G Q P⌊K A A P S V⌊T L F P 125
126 P S S⌊E E L⌊Q A N⌊K A T L V C⌊L⌊I⌊S⌊D⌊F⌊Y P G A V 150
151 T⌊V⌊A W⌊K⌊A⌊D S⌊S P V⌊K⌊A⌊G⌊V⌊E T⌊T⌊T P⌊S K⌊Q⌊S⌊N 175
176⌊N K Y⌊A A⌊S⌊S⌊Y⌊L⌊S⌊L⌊T P⌊E⌊Q⌊W⌊K S⌊H R⌊S Y S C Q 200
201 V T H⌊E G⌊S T V E K T V A P T E C S C

Sequence coverage: 46%

Figure 2. Lc subunit of SILuLite mAb. (A) ETD MS^2 spectrum (50 microscans averaged) of the Lc subunit of SILuLite. The 24+ precursor was *m/z* selected using the quadrupole (leftover precursor highlighted in pink). Portions of the mass spectrum are magnified 5x to visualize product ions. The inset shows the high degree of overlap of the highly charged ion clusters in the *m/z* 960–970 region. (B) The graphical fragmentation map includes only 3 *c/z* complementary ion pair.

To further extend the sequence coverage and enable identification of low S/N, large ETD product ions not detected in MS^2 experiments, PTCR can be included as a part of the targeted method. In this way, two consecutive ion-ion reactions are performed in the high-pressure cell of the dual-pressure quadrupole linear ion trap, as illustrated in Figure 3. First, ions of a single precursor charge state are *m/z* selected with the quadrupole *m/z* analyzer and delivered to the QLT where electron transfer dissociation is performed generating both ETD fragment ions and intact charge reduced (ETnoD) products (MS^2 event); then, product ions are selected in the QLT using a large *m/z* isolation window (1,500–1,800 *m/z* unit-wide, typically centered at *m/z* 1,100), and PTCR reagent ions (perfluoroperhydrophenanthrene anions) are injected into the QLT and used to deprotonate the ETD products (PTCR MS^3 event). Finally, charge-reduced ions are *m/z* analyzed in the Orbitrap at high resolving power using the extended mass range in HMR^n mode (up to *m/z* 8,000).

The PTCR duration was chosen to distribute the product ion population throughout the available mass range without significantly impacting the spectral S/N. Depending on the available upper *m/z* analysis limit, some optimization of the PTCR reaction is required. The PTCR reagent target value has to be set in large excess of reagent ions relative to cations (here: 2e6 charges); optimal PTCR duration values typically vary between 20 and 50 ms (here: 40 ms).

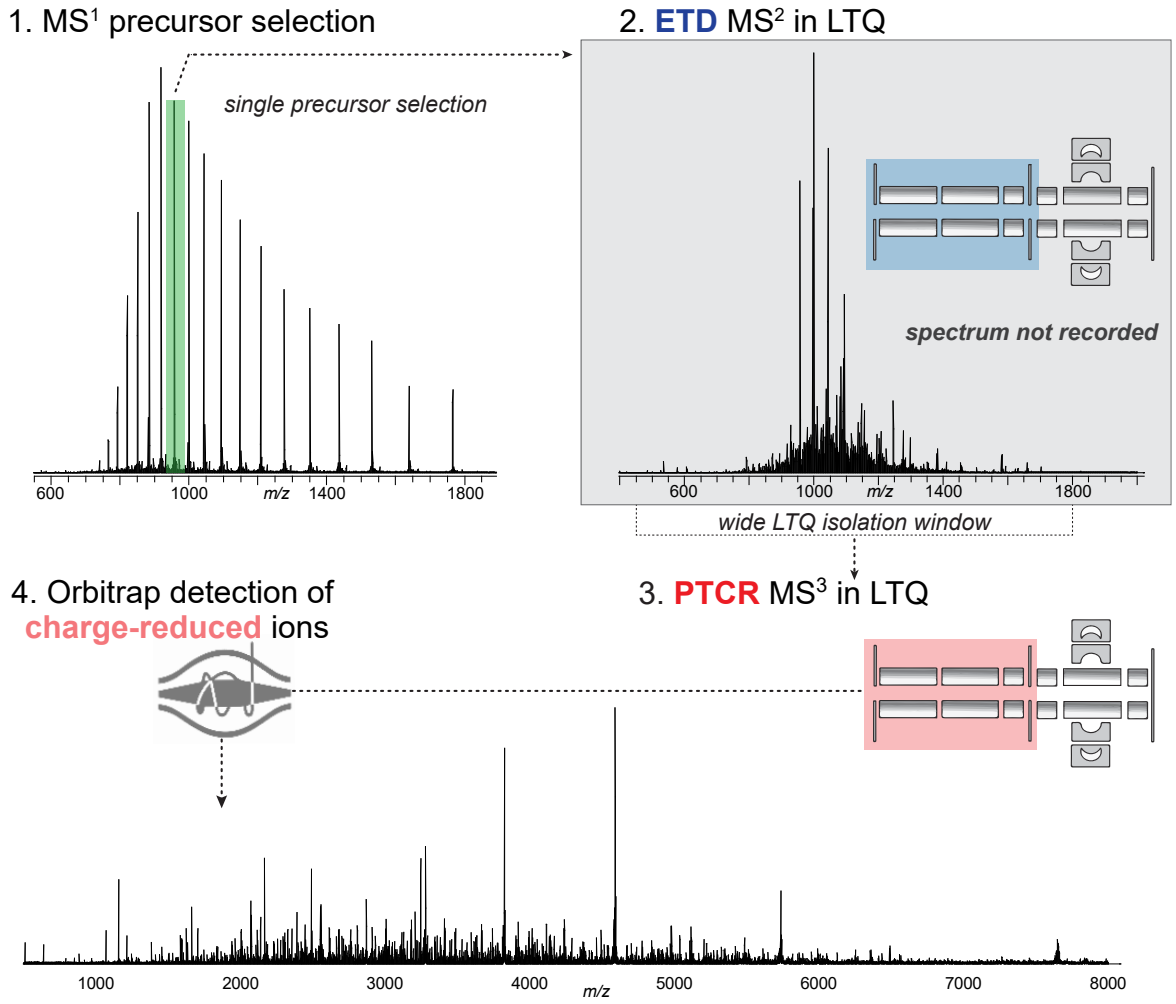


Figure 3. Workflow for ETD MS² – PTCR MS³ experiments. The ETD MS² experiment is performed starting from a single precursor charge state as in the standard targeted method for MS²-level acquisition (steps 1 and 2). This time the ETD reaction is followed by the selection of all ETD products using a wide *m/z* isolation window; these cations are reacted with PTCR reagent anions in a second ion-ion reaction that also takes place in the high-pressure cell of the LTQ (PTCR MS³ event, step 3). Finally, all charge-reduced species are transferred to the Orbitrap mass analyzer to record a mass spectrum with high resolving power and accuracy (step 4).

As shown in the zoomed-in view of the ETD MS² – PTCR MS³ spectrum in Figure 4, most product ions have charge states spanning from 1+ to 4+. Charge-reduced species are still present, but these do not substantially interfere with the detection of N- and C-terminal product ions. The corresponding graphical fragmentation map generated using BioPharma Finder software reveals 53 complementary *c/z*-ion pairs (versus the 3 identified in the corresponding ETD MS² spectrum), thus drastically increasing the sequence assignment confidence. In addition, the charge reduction reaction led to 18% improvement in sequence coverage of the SiLuLite mAb Lc subunit, reaching 64% backbone cleavage assignments in a single LC run. Notably, the RMS mass accuracy for product ion mass assignment was 3.9 ppm despite the use of an analysis *m/z* range, which extended up to *m/z* 8,000 (Table 3).

Finally, Figure 5 clearly demonstrates the increase in the average mass of identified product ions: the advantage of PTCR-based results is particularly noticeable for the mass bins between 10 and 15 kDa (11 vs. 56 unique identified fragments for ETD MS² and ETD MS² – PTCR MS³, respectively) and 15–20 kDa (33 unique product ions identified exclusively using PTCR). Notably, all these advantages are obtained without changing the total analysis time. Both the MS² and MS³ experiments are compatible with chromatographic separation of the 25 kDa mAb subunits and the same LC gradient is appropriate for either. Similar improvements in sequence coverage reported here for the Lc are observed for the other subunits: for instance, the Fd' coverage increases from 38% to 52% in transitioning from ETD MS² to ETD MS² – PTCR MS³ experiments (Table 3).

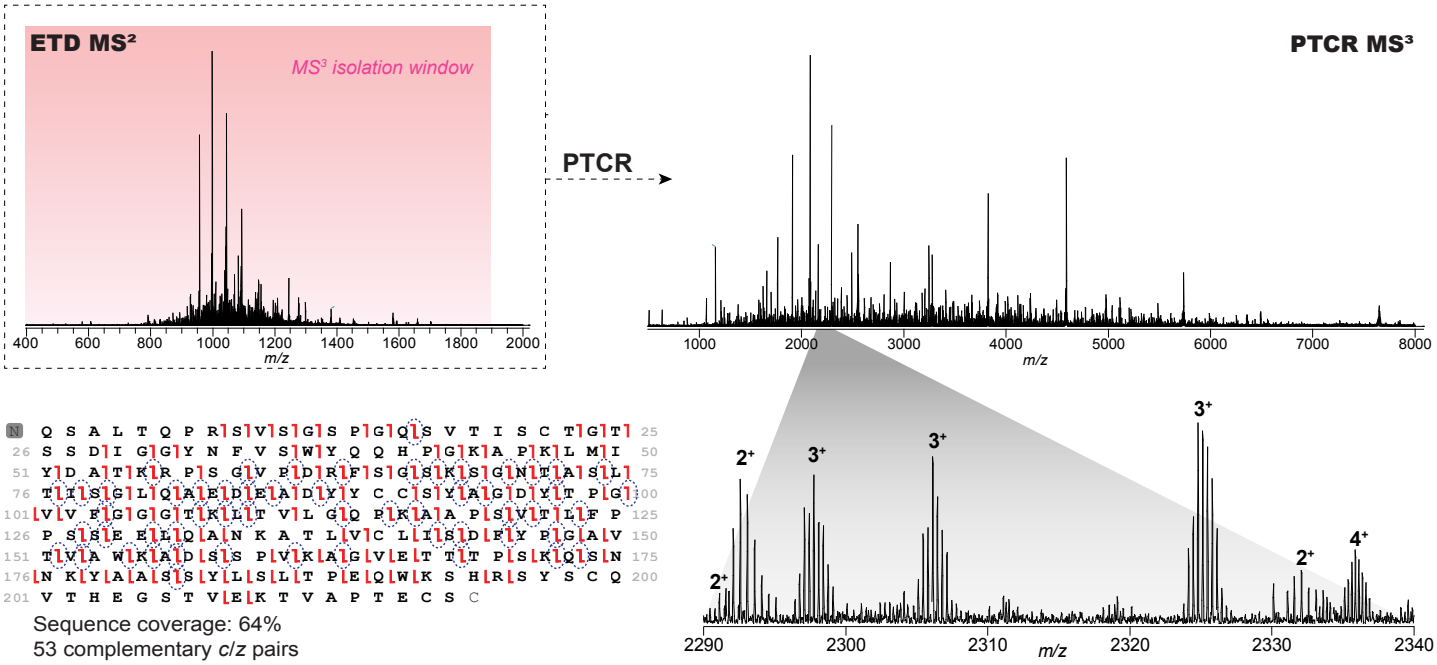


Figure 4. ETD MS² – PTCR MS³ experiment on the Lc subunit of SILuLite. After ETD MS² is performed (2 ms duration) in the LTQ, all ETD products (including ETnoD species) are selected by using a single isolation waveform in the LTQ (isolation width: *m/z* 1,500 units) and subjected to PTCR MS³ (40 ms duration). Charge reduced ETD products are then transferred to the Orbitrap for high resolution/high mass accuracy detection. The inset shows a zoomed-in view of the *m/z* 4,600–4,900 region, where most of isotopic *m/z* peak clusters have charge states from 2+ to 4+ and do not overlap. The graphical fragmentation map includes 53 *c/z* complementary ion pairs.

Table 3. ETD MS² – PTCR MS³ experiment deconvolution results for SILuLite Lc subunit

Experiment	ETD MS²			ETD MS² – PTCR MS³		
Subunit	*Fc/2*	*Lc*	*Fd'*	*Fc/2*	*Lc*	*Fd'*
Sequence coverage	34	46	38	55	64	52
Number of unique matched ions	74	110	100	126	212	152
Number of complementary *c-/z-* pairs	0	3	0	6	53	21
Mass accuracy (ppm RMS)	2.2	2.9	2.8	3.2	3.9	4.3

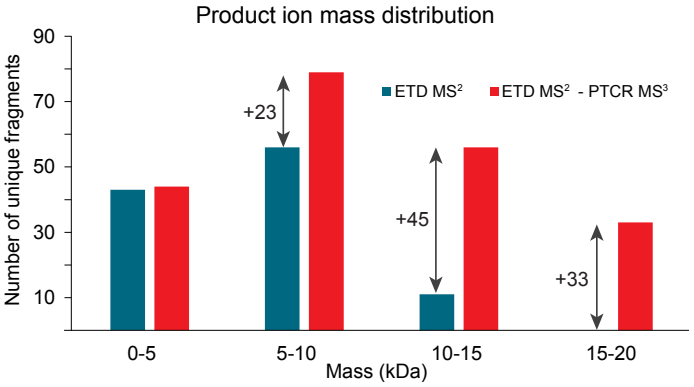


Figure 5. Mass distribution of unique fragment ions identified in ETD MS² (blue bars) and ETD MS² – PTCR MS³ experiments (red bars). PTCR allows the identification of larger product ions otherwise not distinguished from neighboring ion clusters in congested ETD MS² spectra.

A comparison of the insets of Figures 2 and 4 illustrates the reason why PTCR can improve the sequence coverage of these large mAb subunits. The work of any deconvolution or isotope fitting software is dramatically facilitated by the presence of non-overlapping product ions with good S/N and relatively low charge states. Critically, the assignments of large fragments that are usually highly charged and often characterized by low S/N in MS² spectra is made easier in PTCR MS³ spectra. The increased number of complementary ion pairs allows for the confident identification of virtually any chemical modification or single amino acid substitution occurring at any position of the polypeptide chain. The spectral simplification produced by PTCR is reflected also in the parameters used for data analysis, with MS² spectra requiring the application of more stringent Xtract parameters to limit false positive matches resulting from the overlap of highly charged product ion clusters. Conversely, PTCR MS³ spectra exhibit minimal overlap of relatively low charged fragments (see the inset of Figure 4, with ion charge states spanning from +1 to +4). This is in line with what was previously demonstrated for the PTCR-based characterization of standard proteins up to 56 kDa.[4]

Finally, the application of PTCR following ETD requires some adjustments to the data acquisition parameters. The best results in terms of sequence coverage are obtained by increasing ETD reaction time to 3 ms in traditional MS^2 experiments. Conversely, when PTCR MS^3 is applied following ETD MS^2, the duration can be reduced to 2 ms. This reflects that maximizing sequence coverage may benefit from the re-fragmentation of large product ions in MS^2 experiments: these secondary fragmentation events deplete the population of large, first-generation product ions favoring the generation of shorter, less charged second- and higher-generation products that do not typically fall in the immediate vicinity of the precursor *m/z*, where the spectrum is more congested. In this way, signal overlap is mitigated during ETD MS^2 experiments and a larger number of relatively small product ion clusters can be efficiently deconvoluted and identified at the expense of larger primary fragments. In the case of PTCR-based experiments, there is no need to force the ETD reaction to produce shorter product ions, and the best results are obtained by using a 2 ms ETD reaction time[4]; the charge reduction step allows the simultaneous identification of both smaller and larger fragments thanks to their redistribution across a wide *m/z* window that extends up to *m/z* 8,000.

Conclusion

- PTCR is demonstrated to be compatible with the LC separation time scale for middle down mAb characterization.
- Spectral simplification obtained via PTCR facilitates product ion assignment, ultimately leading to increased sequence coverage that can exceed 60% from a single ETD experiment on Orbitrap-based platforms—a result previously achievable only by combining multiple experiments and ion dissociation techniques.
- The application of PTCR allows the identification of large product ions and increases more than ten times the number of identified complementary *c/z* ion pairs in comparison to traditional MS^2 experiments, thus increasing the confidence in sequence assignments.
- This methodology can be easily extended to other orthogonal ion activations available on the Orbitrap Eclipse mass spectrometer, namely CID, HCD, EThcD, and UVPD, further increasing sequence coverage and achieving unparalleled middle-down characterization of the mAb subunits.
- The combination of advanced ion activation methods and gas-phase charge reduction through utilization of PTCR can facilitate deeper insights into the intact structure of mAb drug candidates.

Acknowledgments

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