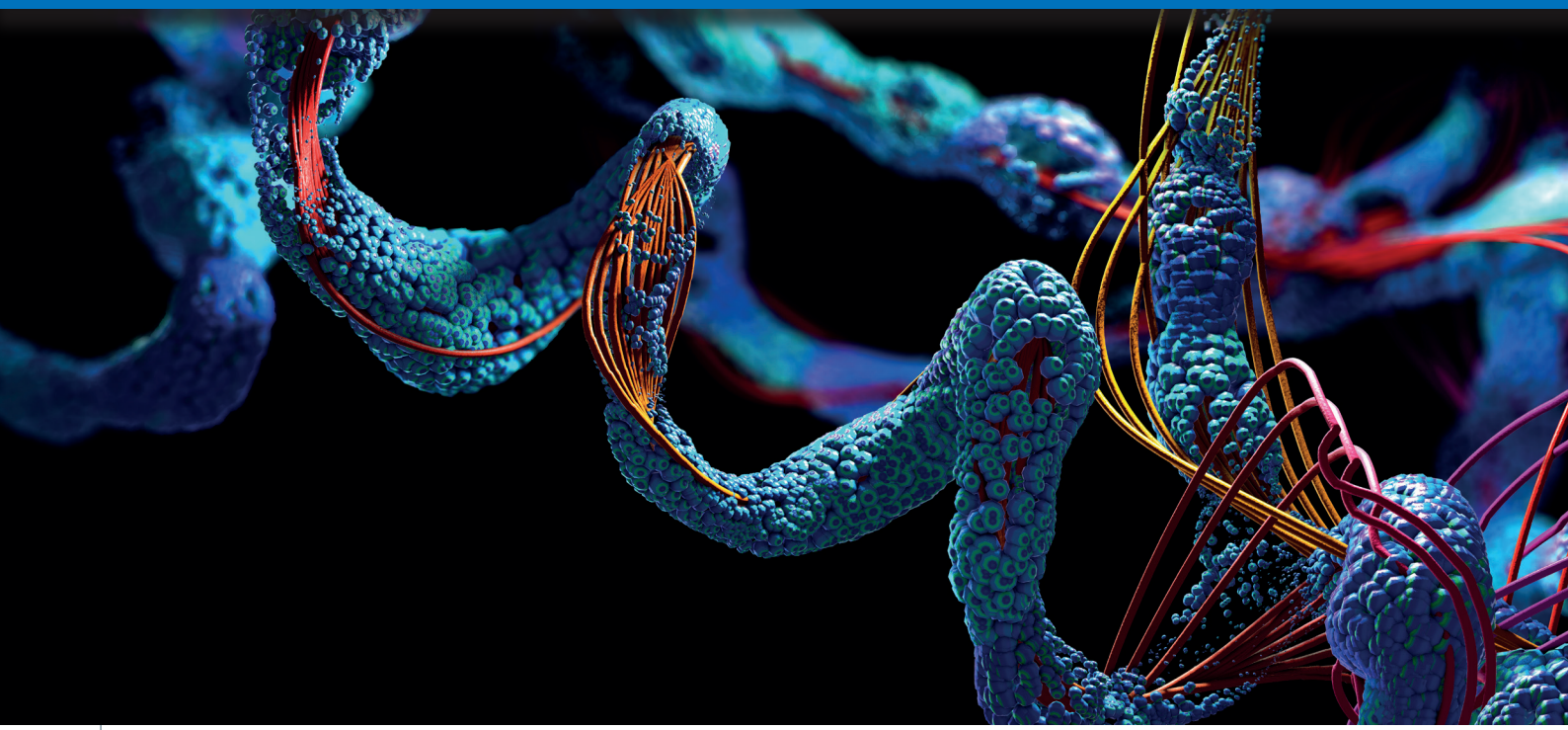

Next-generation MALDI top-down sequencing of protein biotherapeutics – expanding the scope of timsTOF technology

Next-generation MALDI top-down sequencing (MALDI-TDS) improves top-down sequence analysis of biopharmaceutical proteins, as demonstrated in this note for adalimumab subunits, recombinant SARS-CoV-2 S-glycoprotein RBD expression products, and bovine carbonic anhydrase II, a well-characterized model protein.

Abstract

This new method also takes advantage of Trapped Ion Mobility Spectrometry (TIMS), to further enhance MALDI-TDS by adding another dimension of separation in the gas phase.

High confidence N- and C-terminal sequencing specifically are enhanced by combining high resolution (~ 60,000 RP) and <2 ppm mass accuracy delivered by the timsTOF fleX across an ultrawide *m/z* range. This outstanding data quality enables straightforward sequence analysis without the need for charge state deconvolution and warrants safe annotation even for low mass in-source decay (ISD) fragments representing the very near terminal sequence region. TIMS allows for dissection of C- and N-terminal MALDI-TDS spectra resulting in simplified data analysis for individual protein termini, in particular when elucidating unexpected sequence errors or analyzing unknown protein sequences. TIMS also enhances T^3-Sequencing by efficient removal of isobaric

Keywords: Next-generation MALDI-TDS, timsTOF fleX, MALDI, TIMS, top-down sequencing, proteins, biotherapeutics, characterization, biopharma

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background yielding cleaner MS/MS spectra for additional confirmation of the terminal sequence regions.

Next-generation MALDI-TDS adds unique top-down sequencing capabilities to the analytical toolbox making timsTOF fleX an ideally suited platform for in-depth characterization of protein biotherapeutics by providing ultimate ESI and MALDI performance on a single instrument.

Introduction

MALDI top-down protein sequencing has been broadly applied to the characterization of biotherapeutic proteins and delivers confirmation of primary sequences and protein terminal status, such as N-terminal pyroglutamylation or C-terminal lysine loss [1]. It also has been applied successfully to localize glycosylation or PEGylation sites and has provided high sequence coverage to facilitate curation of erroneous reference sequences. [2-6]

Bruker timsTOF fleX enables next-generation MALDI top-down sequencing at ultrahigh resolution (RP 60,000) and previously unseen mass accuracy (<2 ppm) yielding enhanced level of confidence in terminal protein sequencing. Trapped Ion Mobility Spectrometry (TIMS) has the potential to further enhance MALDI-TDS as it allows to dissect C- and N-terminal TDS spectra and, therefore, is of great benefit to simplify data analysis when elucidating unexpected sequence errors or unknown protein sequences. Furthermore, TIMS enhances T^3-Sequencing, i.e. CID-MS/MS analysis of selected ISD fragments for additional confirmation of the terminal sequence, by efficient removal of isobaric interferences yielding cleaner MS/MS spectra.

To highlight the outstanding instrument performance delivered by timsTOF fleX, we present here next-generation MALDI-TDS data obtained from bovine carbonic anhydrase II, a well characterized 29 kDa protein. Furthermore, we discuss various application examples (adalimumab subunits; recombinant SARS CoV-2 S-glycoprotein RBD HEK293 expression product) illustrating the instrument´s unique capabilities for next-generation MALDI top-down sequencing of protein based biotherapeutics.

Bruker timsTOF fleX, due to its ESI/MALDI dual ion source allowing for seamless switching between ionization modalities, provides relentless access to thorough LC based ESI workflows as well as fast, LC-free MALDI analyses. In combination with Trapped Ion Mobility Spectrometry (TIMS) as an additional dimension of separation, this makes the timsTOF fleX a uniquely versatile instrument platform covering a wide range of biopharma applications at an ultimate level of performance.

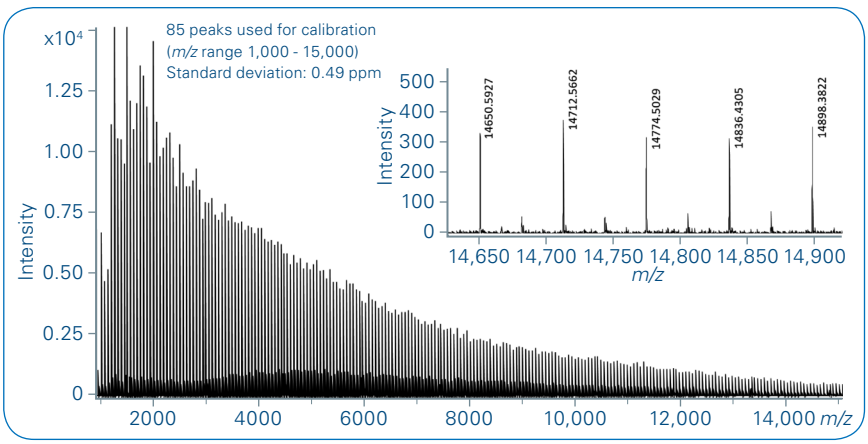

Figure 1. LDI-MS spectrum of red phosphorous used for broadband m/z *calibration.*

Experimental

MALDI sample preparation

Carbonic anhydrase II (CA II, bos taurus) was dissolved in 0.1% TFA (final concentration 100 pmol/μL).

Adalimumab subunits were obtained enzymatically with FabRICATOR (Genovis) and TCEP reduction. 50 μg antibody digest were LC separated and subunits collected in Eppendorf tubes; fractions were concentrated to a final volume of 10-20 μL.

SARS-CoV-2 S-glycoprotein receptor binding domain (RBD) expressed in HEK293 cells [7] was treated with PNGase F (Promega) and SialEXO (Genovis) to remove N-glycan heterogeneity and sialic acids from O-glycans, respectively. Disulfide bridges were reduced using DTT. For more details see [2,3].

Microliter aliquots of protein sample solutions were spotted on a Bruker MTP Anchorchip 384 (CA II; SARS CoV-2 RBD) or MTP BigAnchor 384 MALDI plate (adalimumab subunits) to yield approximately 20-25 pmol of protein per spot, and were dried down under desiccator vacuum. SARS-CoV-2 RBD samples were zip-washed on target with 0.8 μL cold wash buffer (1% TFA, 10 mM $NH_4H_2PO_4$). SDHB MALDI matrix solution (0.5 μL, 25 g/L in 50% ACN, 0.1% TFA) was added to the dry samples, and spots were allowed to crystalize at room conditions.

One microliter of a suspension of crushed red phosphorous in acetone was spotted on a separate plate position as *m/z* calibrant.

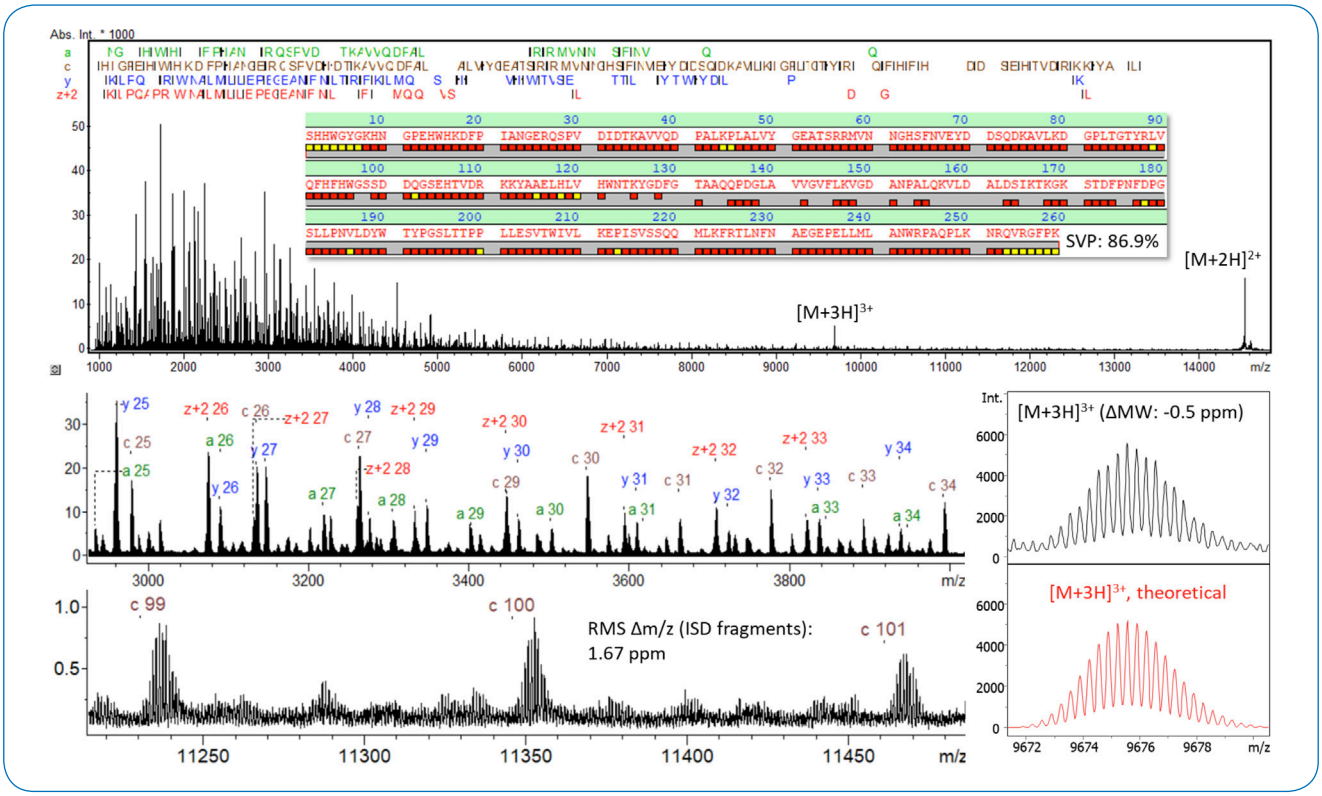

Figure 2. Next-generation MALDI-TDS spectrum of 29 kDa bovine carbonic anhydrase II. Top: Total view on sequence annotated spectrum. Sequence view inset displays sequence validation percentage (SVP) achieved (86.9%). Red bricks indicate amino acids explicitly assigned by respective ISD fragments. Yellow bricks indicate implicite assignments. Upper brick row refers to assignments based on N-terminal ISD fragments of a- and c-type, lower brick row refers to assignments based on C-terminal ISD fragments of y- and z+2-type. Center and bottom right: Zoomed view on 3+ intact mass ion signal detected in the MALDI-TDS spectrum at a resolution of R=60,000. High match quality with theoretical isotope pattern documents outstanding data quality delivered by the timsTOF fleX instrument. Center and bottom left: Zoomed view on various ISD fragment m/z *regions illustrating high quality of sequence annotation.*

Data acquisition

MALDI-TDS spectra were acquired in positive ion mode on a Bruker timsTOF fleX instrument controlled by timsControl 2.0 software. The ion source pressure was adjusted to 1.8 mbar. Data were acquired in the *m/z* range 1,000 – 15,000. A separate method was used for acquisition of MALDI-TDS spectra covering the low *m/z* range below *m/z* 1000.

The instrument´s smartbeam 3D laser was operated at 1 kHz repetition rate using the application profile "MS dried droplet". Spectra were accumulated from up to 30,000 laser shots rastering the outer rim of the sDHB matrix (500 – 1000 shots per raster position).

CID-MS/MS spectra of selected MALDI-ISD fragments (T^3-Sequencing, [8]) were acquired in positive ion MS/MS mode using collision energies between 50 and 120eV, depending on the parent *m/z*.

For MALDI-TIMS-TDS analyses, custom mode at 300 ms TIMS cycle ramp time and 1/K0 gradients optimized for individual samples was used. MALDI-TIMS-T^3-Sequencing spectra were acquired with 800 ms IMS cycle ramp time using narrow $1/K_0$ gradients optimized for individual targeted precursors.

Data processing and analysis

Data were processed in Bruker´s DataAnalysis 5.3 software applying Savitzky Golay smoothing (6pt, 1-2 cycles) and SNAP 2.0 monoisotopic peak picking (Quality factor threshold: 0.3; S/N threshold: 1; rel./ abs. intensity thresholds: 0). BioPharma Compass® 2021b and Biotools 3.2 SR7 software (both Bruker GmbH & Co. KG) were used for data interpretation.

Results and Discussion

Robust broadband *m/z* calibration using red phosphorous as a reference substance

To fully exploit the instrument´s outstanding mass accuracy across a wide mass range for MALDI-TDS measurements, broadband mass calibration is of crucial importance. Red phosphorous represents a perfectly well suited calibrant for that purpose producing monoisotopic P_n cluster ion signals across a wide *m/z* range (*m/z* 100 - 15,000) allowing for convenient and robust mass calibration (Figure 1).

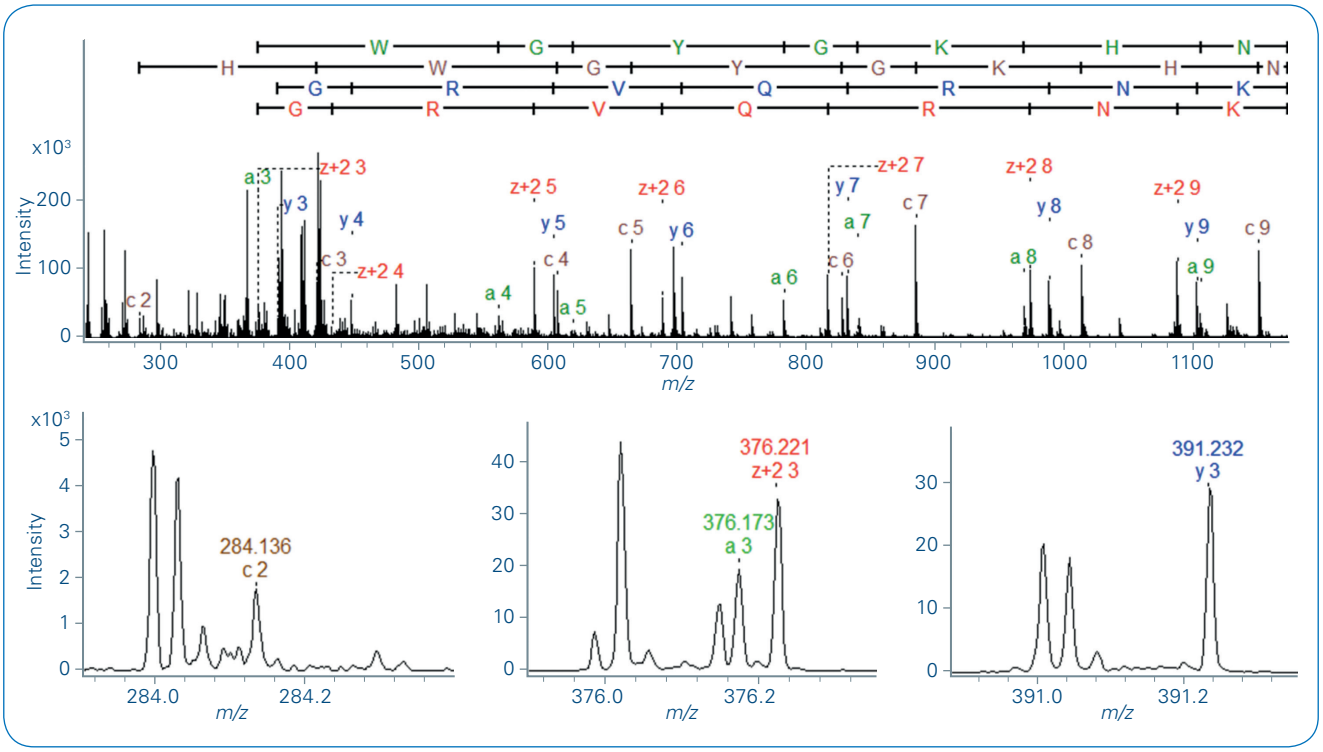

Figure 3. Low-mass MALDI-ISD spectrum of bovine CA II. High resolution maintained in the low m/z *region allows for highly confident sequence verification of near terminal amino acid residues.*

Next-generation MALDI-TDS performance characteristics of timsTOF fleX

Initially, we analyzed bovine carbonic anhydrase II (CA II), a well characterized 29 kDa standard protein (Figure 2). Next-generation MALDI-TDS data acquired on timsTOF fleX provide, in one and the same spectrum, accurate intact mass information and highly confident C- and N-terminal sequence readout. Multiply charged molecular ions and singly charged ISD fragments are isotopically resolved (60,000 RP) throughout the entire *m/z* range of interest, rendering straightforward data interpretation and visual validation without the need for charge deconvolution. Outstanding mass accuracy (typically <2 ppm) is achieved for both, intact mass signals and ISD fragments. Matching the data against the amino acid sequence of CA II yielded a sequence validation percentage (SVP) of 86.9%.

High resolving power is maintained on timsTOF fleX in the low *m/z* range allowing for acquisition of meaningful MALDI-TDS data in the critical mass range below *m/z* 1000. Short length MALDI-ISD fragments appear well resolved from complex chemical background and, hence, enable sequence verification down to the very terminal amino acid residues (Figure 3).

Engaging TIMS further enhances MALDI-TDS by providing additional separation space and, thus, reducing spectral complexity (Figure 4). In the resulting MALDI-TIMS-MS heatmap (Figure 4A,), singly charged ISD fragments appear as the dominating charge state accompanied by a lower abundant population of doubly charged ISD fragments. The region accommodating singly charged ions separates into two sub-regions representing C- and N-terminal MALDI-ISD fragments, respectively, allowing for dissection of terminus-specific MALDI-TIMS-TDS spectra (Figure 4B, C). These spectra facilitate simplified data interpretation for individual protein termini, which is particularly beneficial when elucidating unexpected sequence errors or analyzing unknown protein sequences.

Total SVP achieved for bovine CA II in MALDI-TIMS-TDS based on assignment of 1+ and 2+ ISD fragments was 72% (sequence annotation of 2+ ISD fragments not shown here).

The additional separation space provided by TIMS also enhances T^3-Sequencing [8], i.e. pseudo-MS^3 analysis of selected MALDI-ISD fragments by CID-MS/MS (Figure 5). TIMS enabled efficient separation of N-terminal ISD fragment c_{18} from an isobaric ISD fragment and, thereby, allowed for acquisition of an interference-free T^3 spectrum yielding additional unambiguous confirmation of the N-terminal protein sequence from serine residue 1 onwards.

Rapid sequence verification of Adalimumab subunits by next-generation MALDI-TDS

Adalimumab subunits were generated by FabRICATOR digest and reduction with TCEP (no deglycosylation; G0F is the major glycoform) followed by LC separation and fraction collection. In case of more complex glycosylation, removal of N-glycans by PNGase F prior to MALDI-TDS analysis is recommended.

Resulting MALDI-TDS data (Figure 6) confirmed the expected protein sequences of all 3 adalimumab subunits based on intact mass measurement at an accuracy level of ≤ 0.5 ppm ($[M+2H]^{2+}$) and sequence readout from matching C- and N-terminal ISD fragments yielding sequence validation percentages in the 68 - 83% range for the individual subunits. Further-

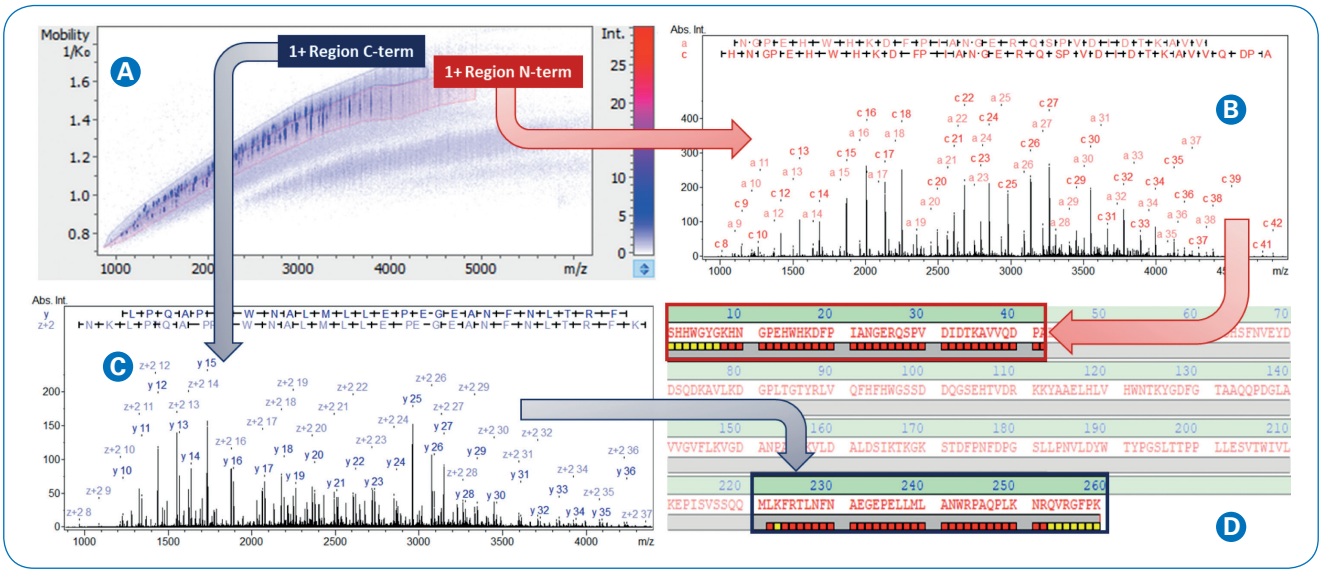

Figure 4. MALDI-TIMS-TDS analysis of CA II. In the resulting MALDI-TIMS-MS heatmap (A), singly charged ISD fragments appear further separated into 2 sub-regions dominated by N- and C-terminal fragments, respectively, allowing for dissection of terminus-specific MALDI-TDS spectra ((B), (C)). Terminal sequence readout obtained from these spectra is shown on the protein sequence view (D).

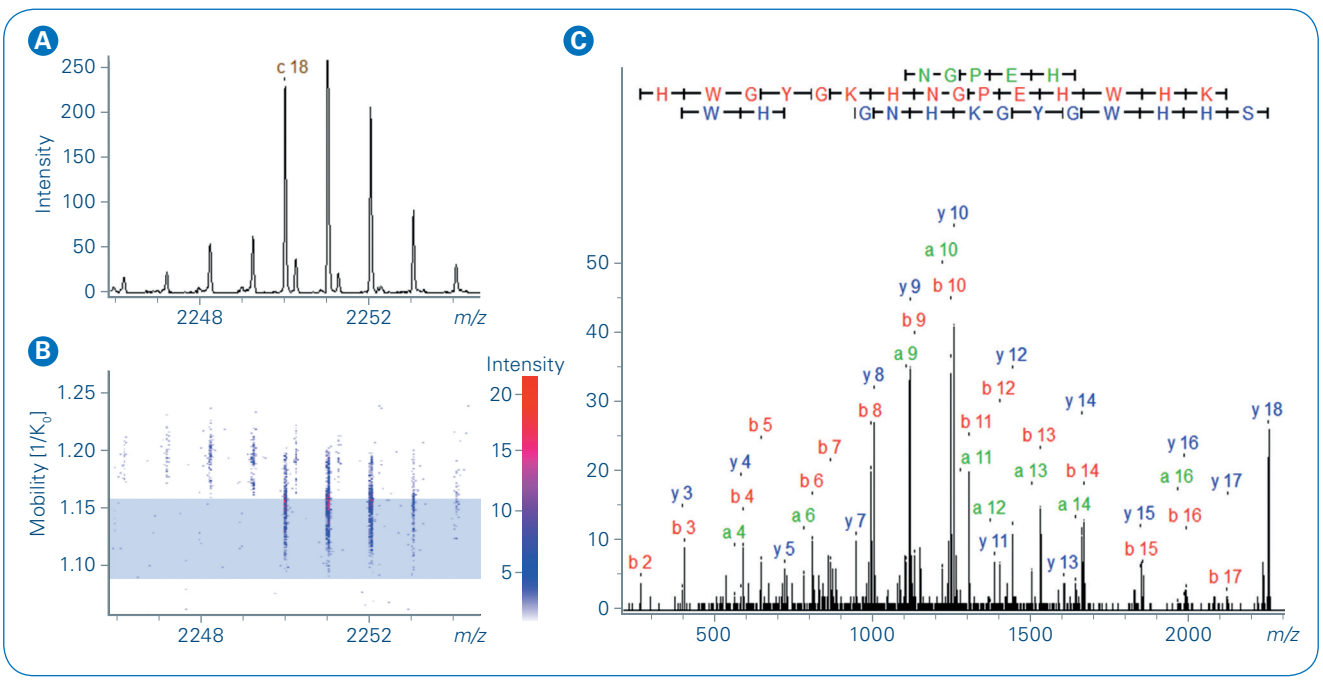

Figure 5. T³-Sequencing analysis of CA II enhanced by TIMS: (A) MALDI-TDS spectrum zoomed on N-terminal ISD fragment c_{18} overlapping with a near-neighbor signal. (B) MALDI-TIMS-MS heatmap showing the ion mobility separation of N-terminal MALDI-ISD fragment c_{18} from the overlapping signal. (C) Interference-free MALDI-TIMS-T³-Sequencing spectrum of N-terminal ISD fragment c_{18} extracted from the mobility region labeled blue in panel B.

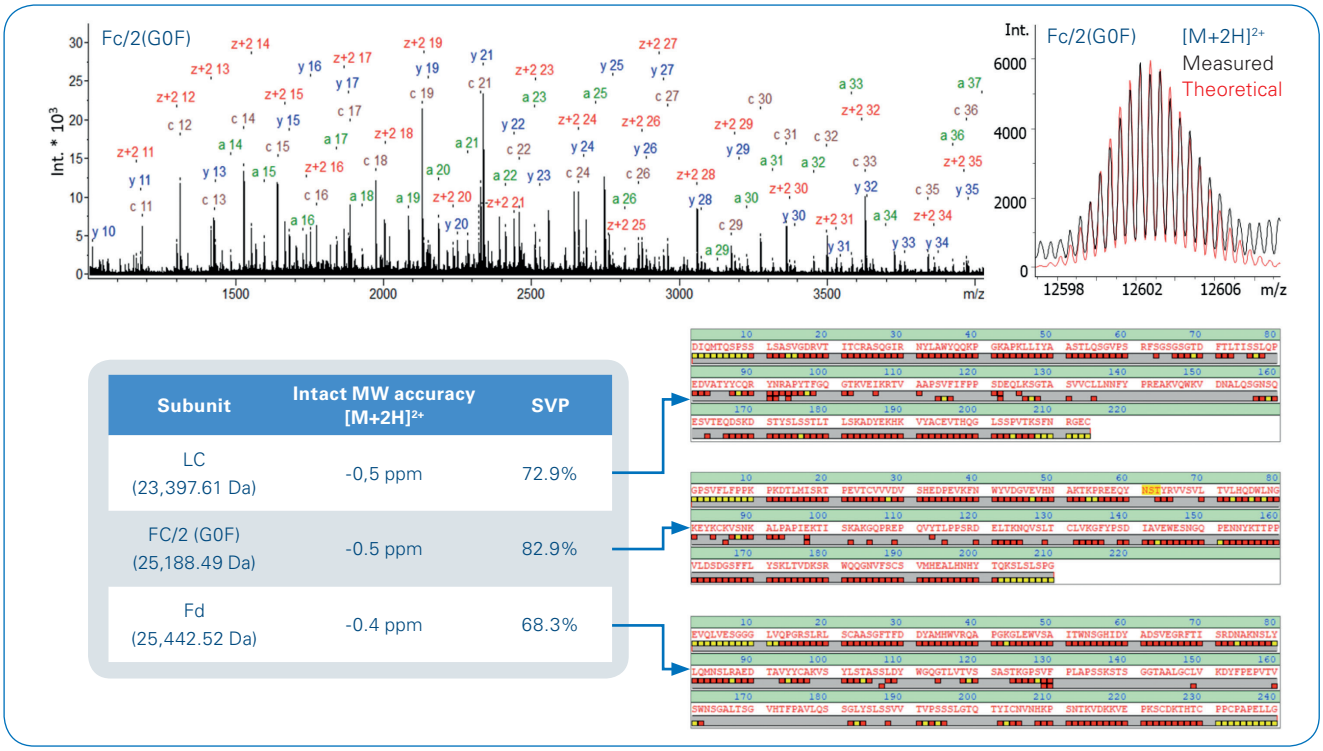

Figure 6. Next-generation MALDI-TDS results obtained from adalimumab subunits. Example spectrum shown as zoomed view in the top panel originates from subunit Fc/2 considering major glycoform G0F.

more, MALDI-TDS data confirmed C-terminal lysine-loss of the Fc/2 subunit, and LC´s and Fd´s N/C-termini to be free of modifications.

It is particularly worth to note that all information was extracted from a minimum amount of data, i.e. from one single MALDI-TDS spectrum per subunit, which further highlights the speed, high data quality and wealth of information provided by the MALDI-TDS approach in general and the timsTOF fleX instrument in particular.

Sequence verification and determination of O-glycosylation site occupancy in recombinant SARS-CoV-2 S-glycoprotein RBD

Next-generation MALDI-TDS analysis of recombinant SARS-CoV-2 RBD expressed in HEK293 cells (Figure 7) yielded the following results:

- Presence of core 1 and core 2 O-glycosylation was verified by the appearance of an intact mass doublet peak $[M+2H]^{2+}$ with a characteristic mass distance of 365 Da matching one HexNAcHex group separating a core 1 from a core 2 O-glycan (Figure 7, top).
- Measured intact masses and matching N-terminal ISD fragments (a- and c-type) confirmed the presence of a pyroglutamine residue at the RBD´s N-terminus arising from an unexpected cleavage within the pro-peptide (Figure 7, center). [3]
- Matching N-terminal ISD fragments also provided evidence for Thr-6 as the only O-glycosylation site, the alternative site Ser-8 was not glycosylated. [3]
- MALDI-TIMS-T^3-Sequencing spectra of N-terminal ISD fragment c_7, both from core 1 and core 2 modified RBD, provided further unambiguous evidence for Thr-6 as the active O-glycosylation site (Figure 7, bottom). T^3 spectra show characteristic fragment ions resulting from sequential loss of hexose and N-acetylhexosamine units O-linked to Thr-6. Engaging TIMS enhanced T^3 spectra quality by efficient separation of any unrelated fragments originating from co-isolated isobaric background.

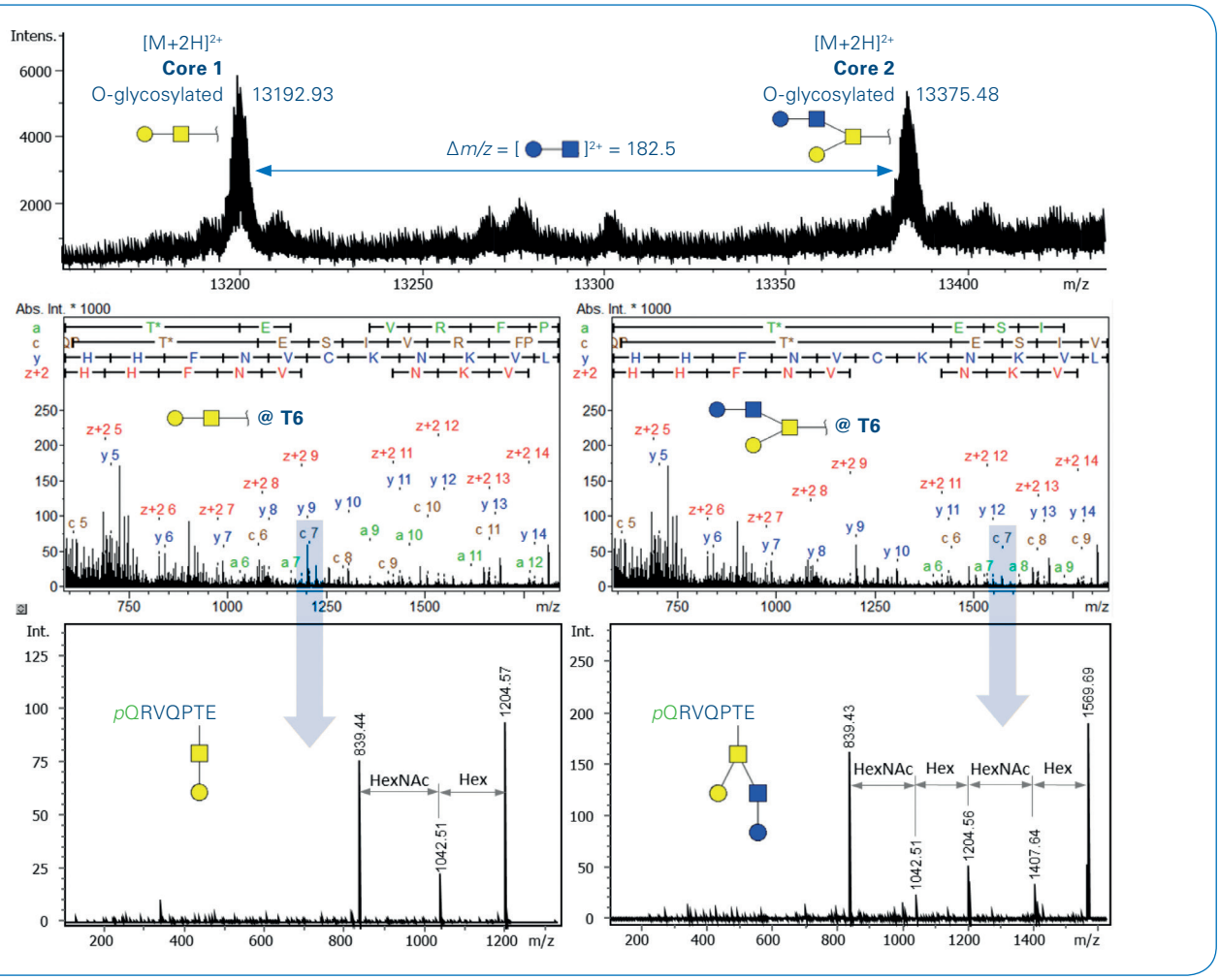

Figure 7. Next-generation MALDI-TDS analysis of recombinant SARS-CoV-2 S-glycoprotein RBD HEK293 expression product. Top: MALDI-TDS spectrum zoomed on intact mass signal region (2+) representing core 1 and core 2 O-glycosylated RBD glycoforms. Center: MALDI-TDS spectrum zoomed on mass region comprising ISD fragments $c_6 - c_8$ when matched against RBD glycoforms carrying either core 1 (center, left) or core 2 O-glycosylation (center right), with Thr-6 as proposed glycosylation site. Bottom: TIMS-T³-Sequencing spectra acquired from N-terminal ISD fragments c_7 providing further evidence for the presence of core 1 and core 2 O-glycosylation at Thr-6.

Conclusion

- Next-generation MALDI-TDS on timsTOF fleX delivers instant information regarding primary sequence, terminal status and near-terminal modifications of protein biotherapeutics.
- Next-generation MALDI-TDS facilitates high-confidence C- and N-terminal sequence readout through UHR-TOF analysis of singly charged MALDI-ISD fragments at high resolution (RP 60,000) and mass accuracy (<2 ppm) without the need for charge deconvolution. Accurate intact mass information is extracted from MALDI-TDS spectra at the same time.
- TIMS provides additional separation space and, therefore, reduces the complexity of MALDI-TDS data. Dissection of C- and N-terminal MALDI-TIMS-TDS spectra enhances data interpretation for individual protein termini, allowing for simplified elucidation of unexpected sequence errors or unknown protein sequences. TIMS also enhances T³-Sequencing by efficient separation of isobaric interferences yielding cleaner MS/MS spectra for enhanced validation of the terminal sequence.
- Next-generation MALDI-TDS adds unique top-down sequencing capabilities to the biopharma application space covered by timsTOF fleX at an unparalleled level of ESI and MALDI performance.

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