

# Characterization of Protein-based Biotherapeutics by TIMS enabled Next-Generation MALDI Top-Down Sequencing

Arndt Asperger, Waltraut Evers, Anja Resemann, Detlev Suckau, Christian Albers, Bruker Daltonics GmbH & Co. KG, Bremen, Germany

## Summary

- Next-Gen MALDI-TDS: Top-down sequencing based on MALDI-ISD taking advantage of timsTOF technology
- MALDI-ISD yields 1+ N- and C-terminal backbone fragments allowing for straightforward sequence readout without charge deconvolution
- Trapped Ion Mobility Spectrometry (TIMS) separates MALDI-ISD fragments according N- or C-terminal origin enabling terminally dissected top-down sequence analysis.
- TIMS enables interference-free T<sup>3</sup>-Sequencing (CID-MS/MS of selected MALDI-ISD fragments) for verification of very terminal sequence regions.
- Next-Gen MALDI-TDS allows for rapid, in-depth characterization of protein-based biologics re. primary sequence, terminal status and near-terminal modifications

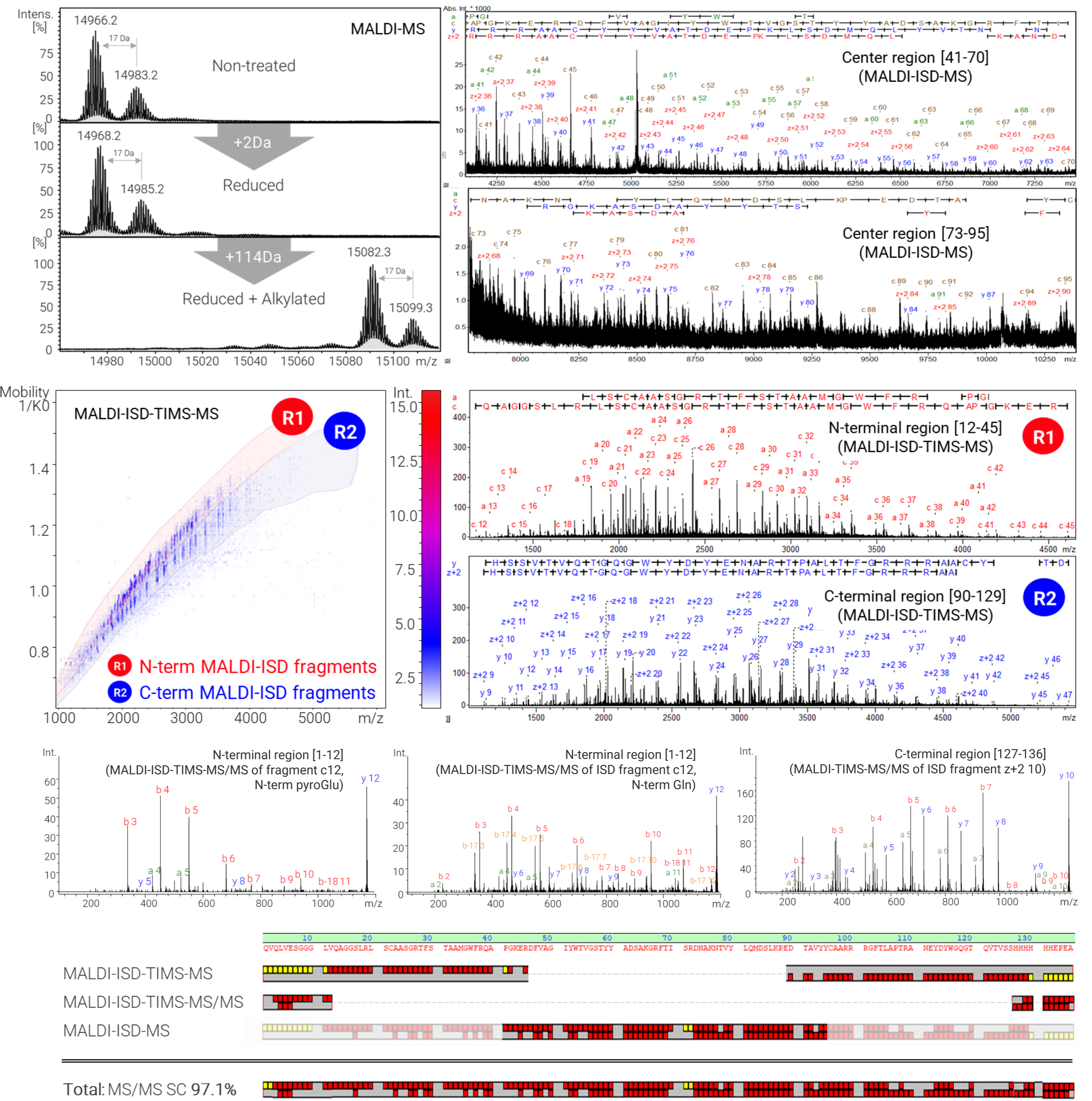
## Methods

**Samples:**  
**Nanobody:** Reduction with DTT; alkylation with IAA; sample clean-up by microdialysis  
**Recomb. SARS-CoV-2-S-glycoprotein-RBD:** Expressed in and purified from HEK293 cells; reduction with DTT; release of N-glycans with PNGaseF (Promega); cleavage of sialic acids with SialExo (Genovis); sample clean-up by on-target washing  
**MALDI preparation:** 10-20 pmol protein per sample spot; MALDI matrix sDHB (Bruker); MALDI plate MTP Anchorchip 384 BC (Bruker); red phosphorus was spotted on a separate spot position as calibrant for instrument m/z calibration  
**Data acquisition:** Bruker timsTOF fleX instrument with dual ESI/MALDI ion source; Positive MALDI ion mode; TIMS in pressure reduced to 1.8 mbar TIMS ramp 300ms; 1/K0 range 0.6 – 1.9  
**Data analysis:** Bruker DataAnalysis (smoothing, baseline, peakfinding), Bruker Biotools (sequence analysis)

## Results II (Biologics applications)

Application I: Complete characterization of a 15 kDa nanobody

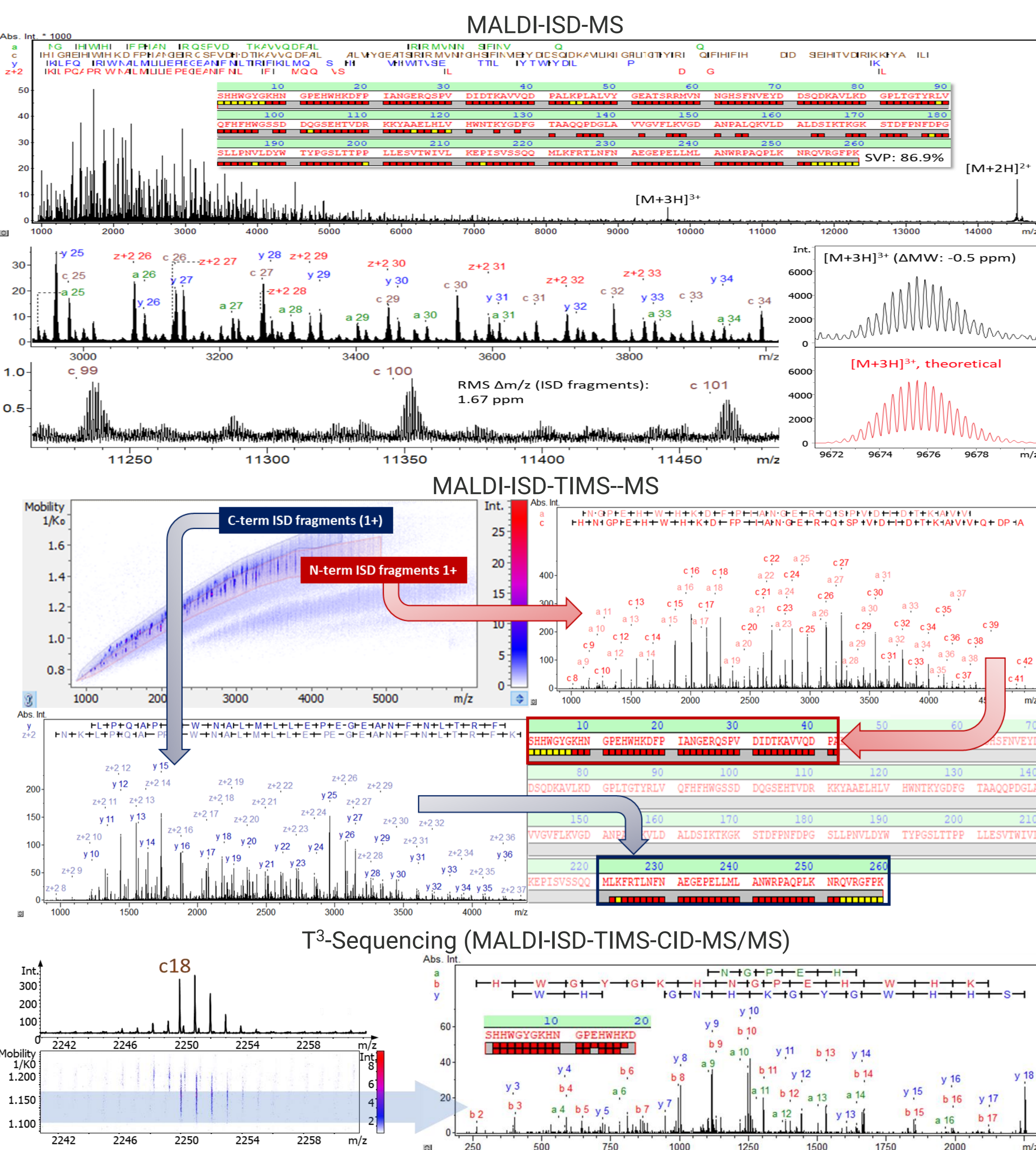
- Next-Gen MALDI-TDS yielded the complete nanobody sequence
  - 136 AA residues
  - Partially Gln->pyroGlu converted N-terminus
  - 2 cysteines linked via disulfide bridge
- Overall MS/MS Seq.Cov. > 97% (132 out of 136 AA residues assigned by fragment ions)
- 20% of the AA sequence was retrieved denovo (due to conflicting/lacking reference data)
- Sequencing result reconfirmed by matching nanobody MW obtained from intact-mass MALDI-MS data ( $\Delta MW < 0.2$  ppm)



## Results I (timsTOF fleX performance characteristics)

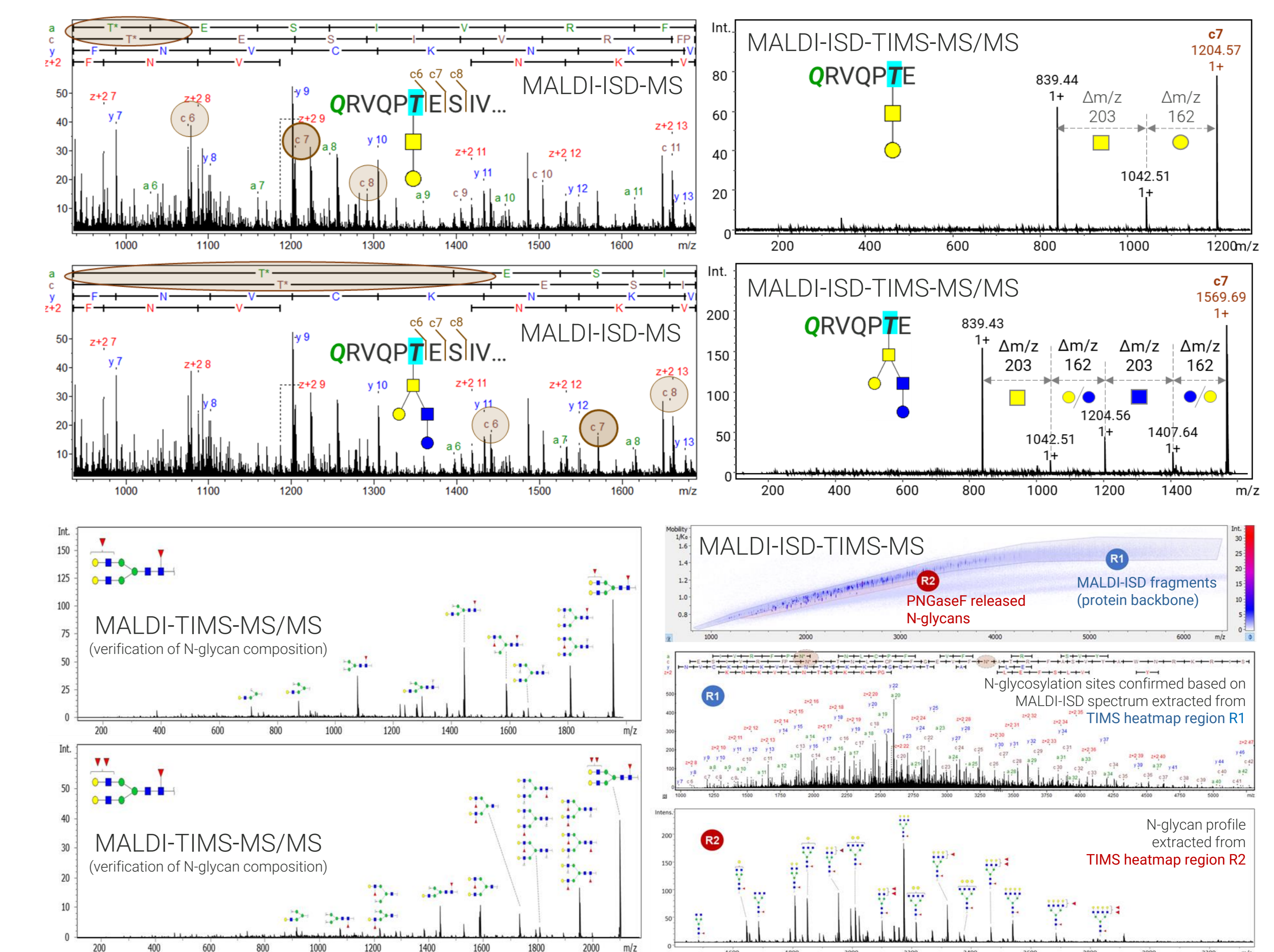
Performance of the timsTOF fleX instrument in Next-Gen MALDI-TDS was investigated using Carbonic Anhydrase II (*bos taurus*), MW 29 kDa, as a standard protein:

- Sequence verification percentage (SVP) achieved by MALDI-ISD-MS: 86.9%
- ISD fragments throughout m/z range 1,000 – 15,000 all isotopically resolved
- RMS  $\Delta m/z$  (across all ISD fragments): 1.67 ppm
- Accurate intact-mass:  $\Delta m/z$  -0.5 ppm ( $[M+3H]^{3+}$  signal)
- TIMS benefits to Next-Gen MALDI-TDS:
  - TIMS separation of ISD fragments according N- or C-terminal origin enables terminally dissected top-down analysis of terminal sequence regions
    - Reduced data complexity
    - Overlaps resolved for isobaric fragments originating from opposite termini
    - Simplified data interpretation in denovo-like sequencing tasks
  - Enhanced T<sup>3</sup>-Sequencing (i.e. CID-MS/MS of selected ISD fragments) of very terminal sequence regions by TIMS separation of co-isolated isobaric background



Application II: Characterization of O- and N-glycosylation in recomb. SARS-CoV-2-S-glycoprotein-RBD expressed in HEK293

- Active site of O-glycosylation pinpointed at position T6 (S8 is not O-glycosylated)
- O-glycosylation site T6 features both, core 1 and core 2 O-glycans
- Active sites of N-glycosylation verified (N14; N26)
- N-glycan profile features highly host-specific multiply fucosylated N-glycan compositions



**Acknowledgement:** We would like to thank Professor Jan Steyaert, Vrije Universiteit Brussel, Belgium, for providing the nanobody sample.

Biologics / timsTOF fleX