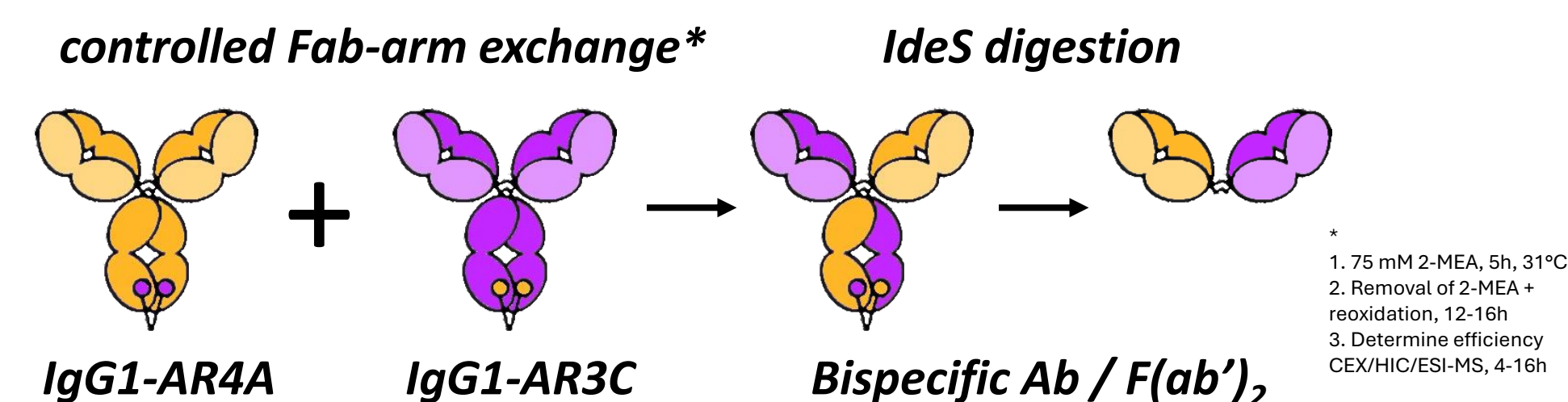


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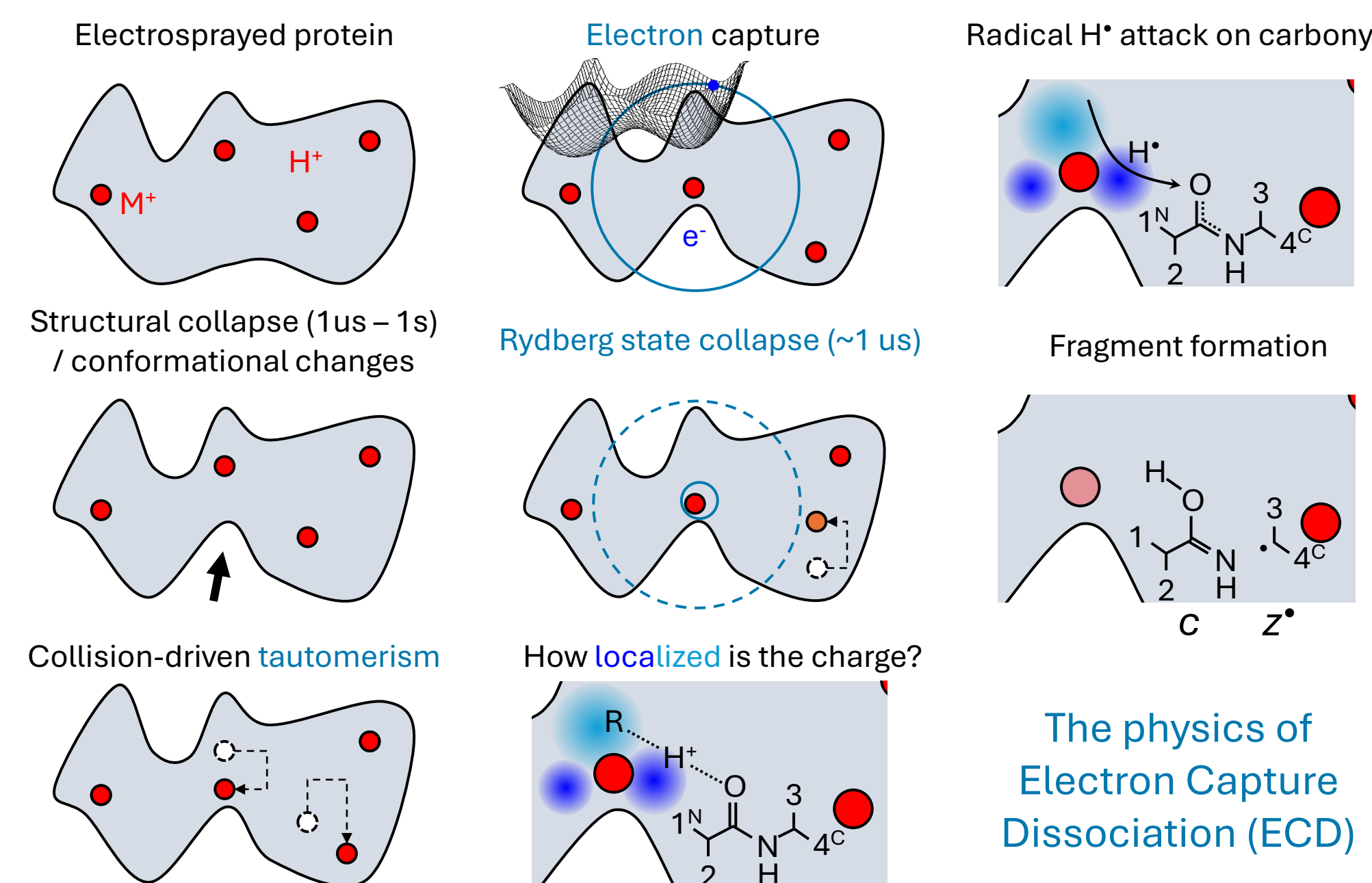
<sup>1</sup>Biomolecular Mass Spectrometry & Proteomics, NL, <sup>2</sup>Netherlands Proteomics Center, NL, <sup>3</sup>Fasmatech, GR, <sup>4</sup>Bruker Daltonics GmbH & Co. KG, D, <sup>5</sup>Bruker Switzerland AG, CH

## Introduction

- The ability of bispecific antibodies (bsAbs) to simultaneously bind two different antigens or two different epitopes on the same antigen makes them highly effective therapeutic agents.



- bsAbs are ideally characterized by top-down MS<sup>n</sup> electron capture dissociation as it can preserve their sequence integrity, explore disulfide connectivity, and map posttranslational modifications.
- Positive charge localization and disulfide bridges focus fragment formation to the bsAbs functional hypervariable regions, especially the Complementarity-Determining Region 3 of the light and heavy chains.



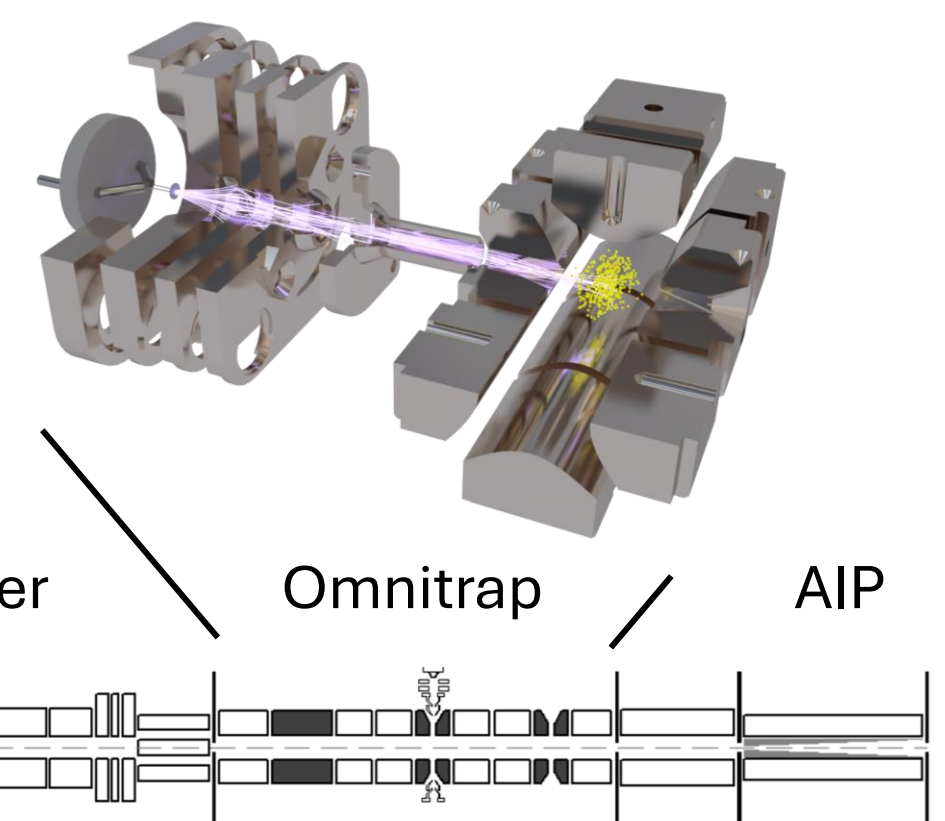
## Methods

- F(ab)<sub>2</sub> and Fabs, ~100 / ~50 kDa fragments containing the bsAbs' hypervariable regions selectively binding to antigens, were produced by enzymatic digestion using the hinge directed proteases IdeS and IgD.
- Electrosprayed F(ab)<sub>2</sub> with charge states spanning the 50<sup>+</sup> to 20<sup>+</sup> range were mass selected, trapped in the Omnitrap™, before being fragmented by ECD and detected.
- The results were processed using DataAnalysis™, OmniScape™, and custom-made analysis tools.

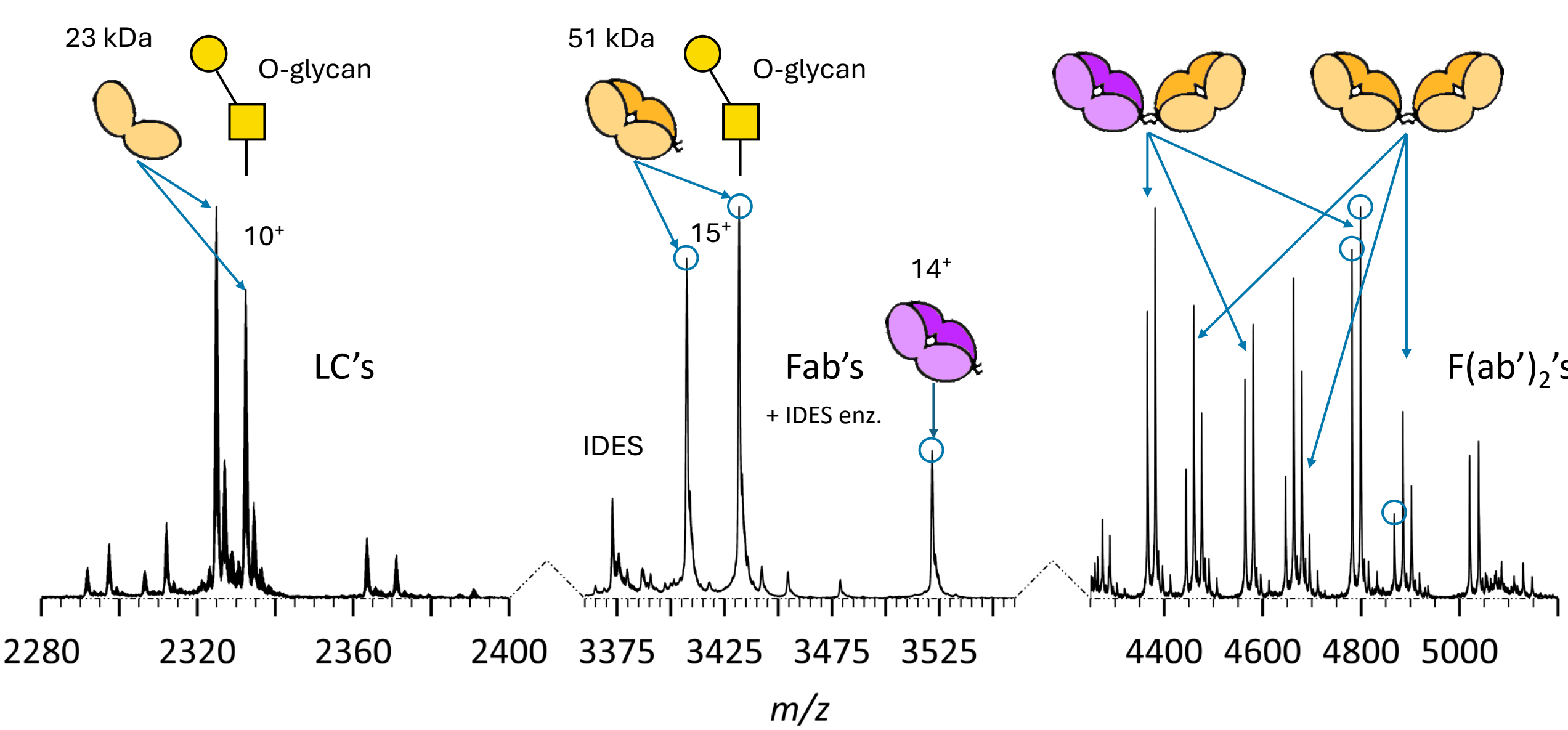
## Instrumentation

- Schematic of a Bruker timsOmni™ IMS Q-Omnitrap™-ToF mass spectrometer. The Omnitrap cell with its electron gun is highlighted.

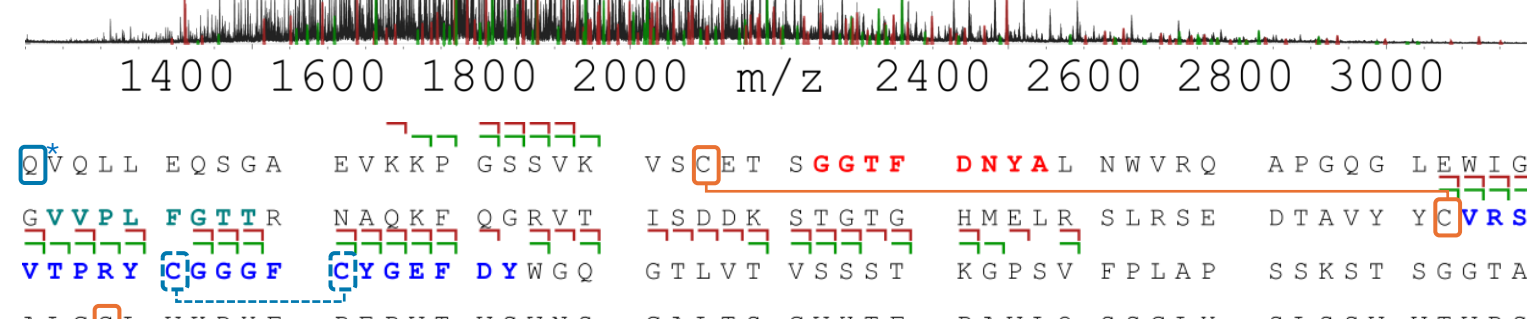
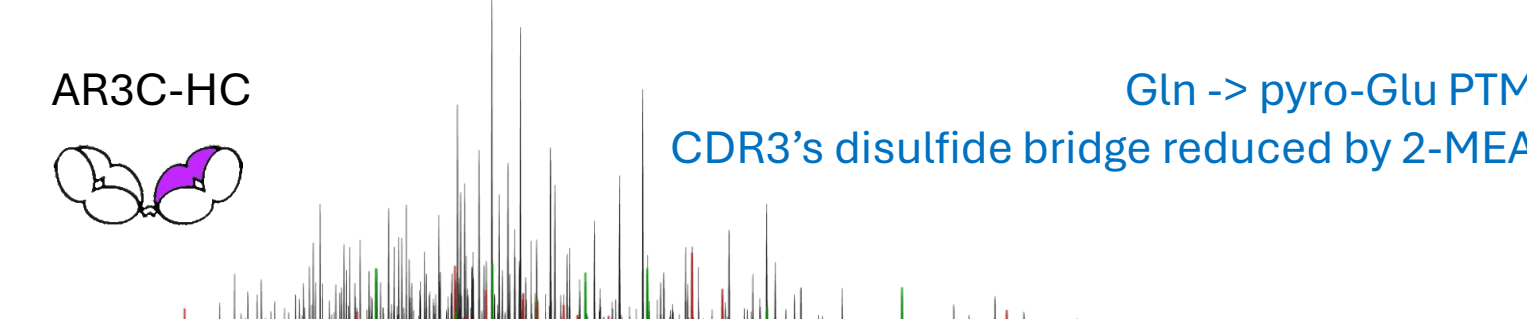
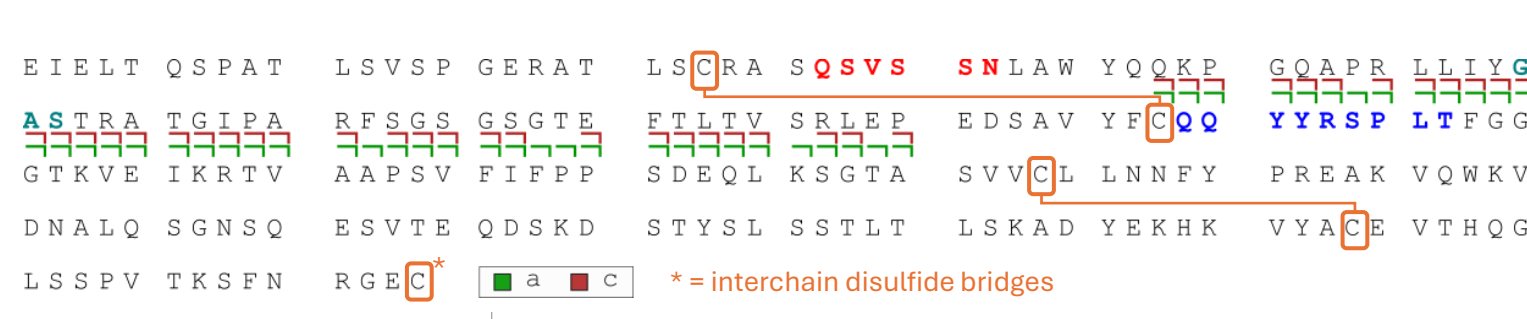
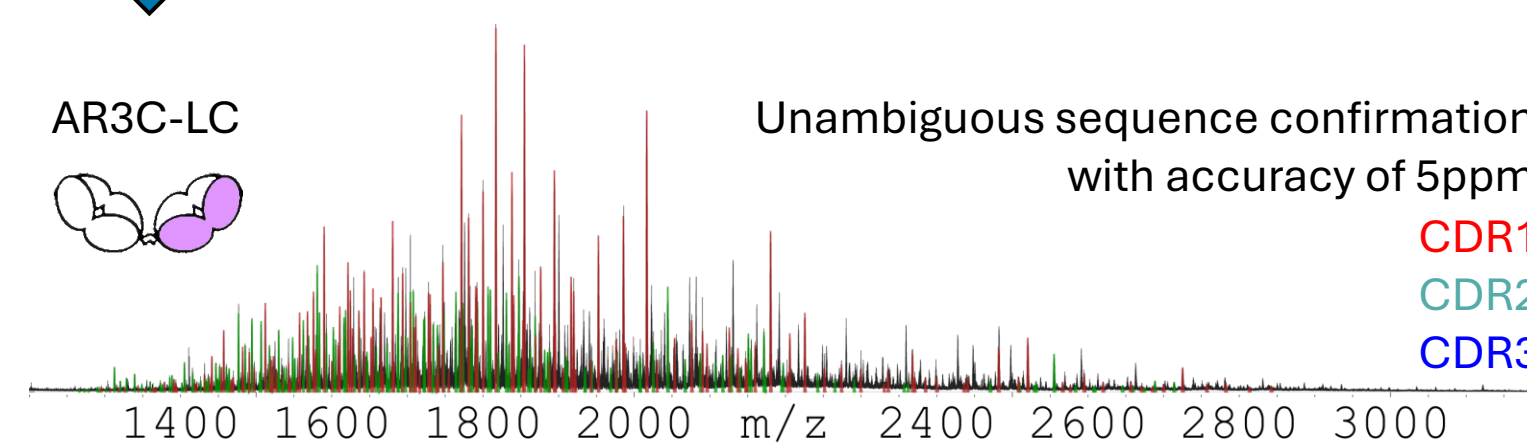
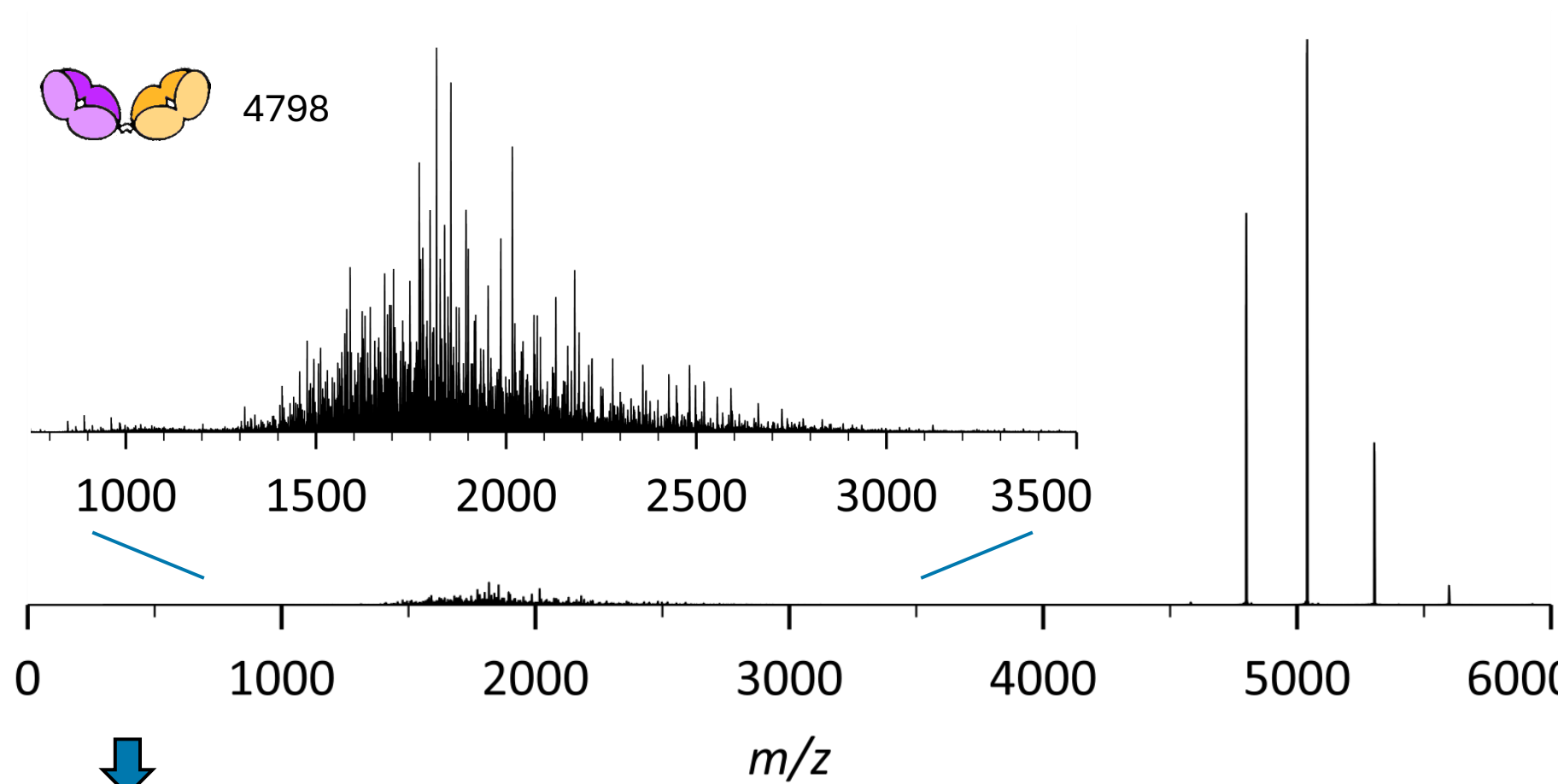
- Nanospray
- isCID 90 eV
- 2s accumulation
- 50ms ECD at ~1 eV KE



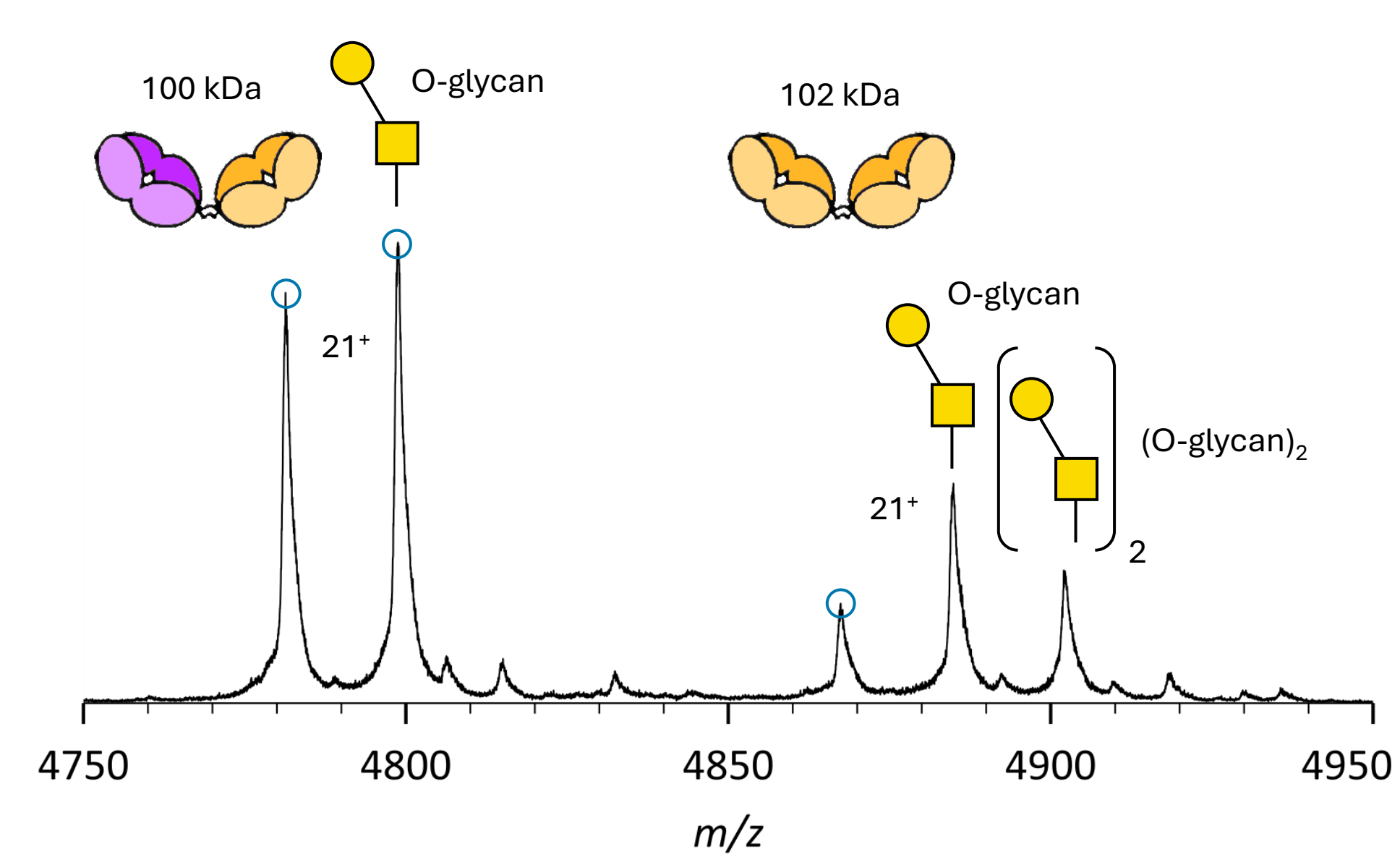
### MS1 spectrum of LAC49 bsAb digest by IdeS (O species selected for ECD)



### MS2 ECD on the LAC49 bsAb's F(ab')2's with one glycan

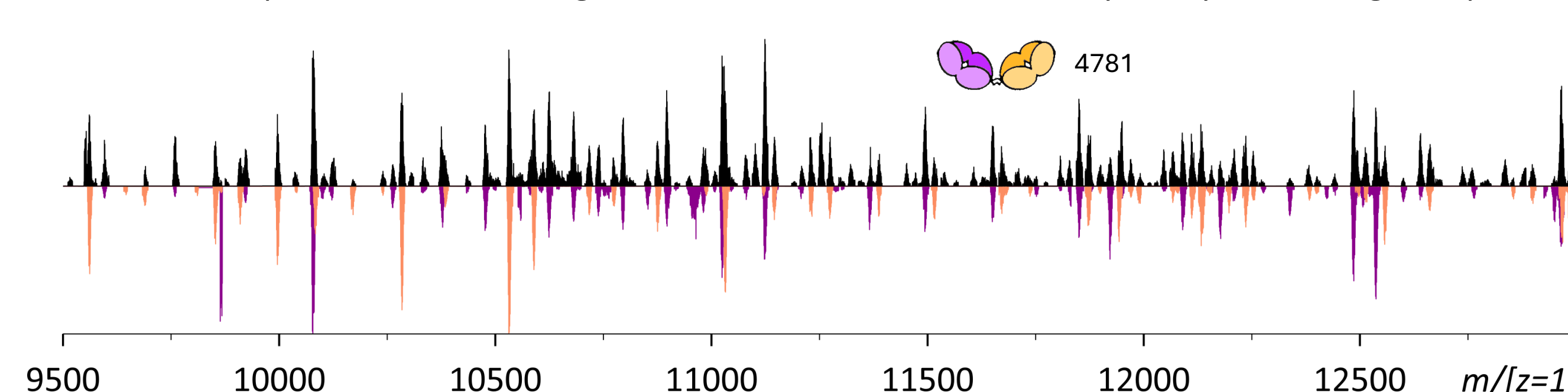


### Clear identification of O-glycosylation on Fabs and F(ab')2's



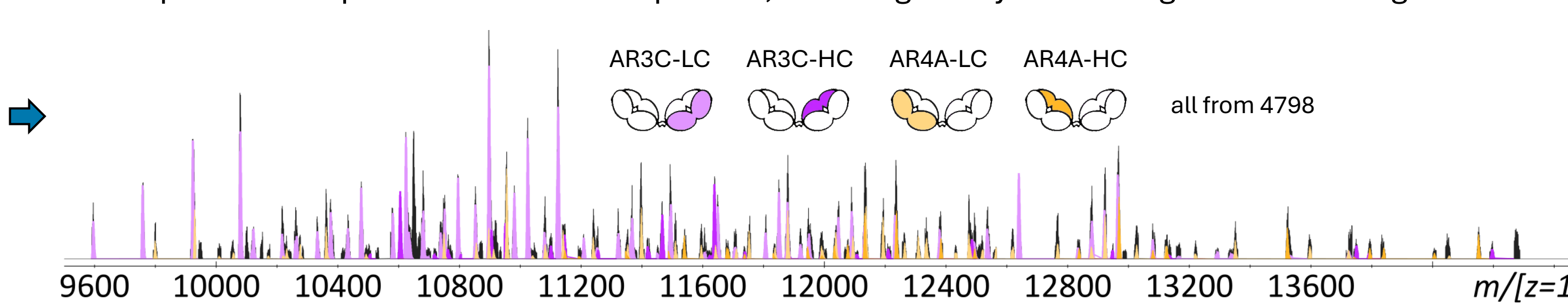
### ECD ion sequence ladders of LAC49 bsAb's and mAb's F(ab')2's and Fabs w/o O-glycan

Easy-to-read ion sequence ladders (E2RISL) generated by the timsOmni, enable direct comparison of the bsAb ECD spectrum with those generated for the subunits, thereby nicely facilitating interpretation.



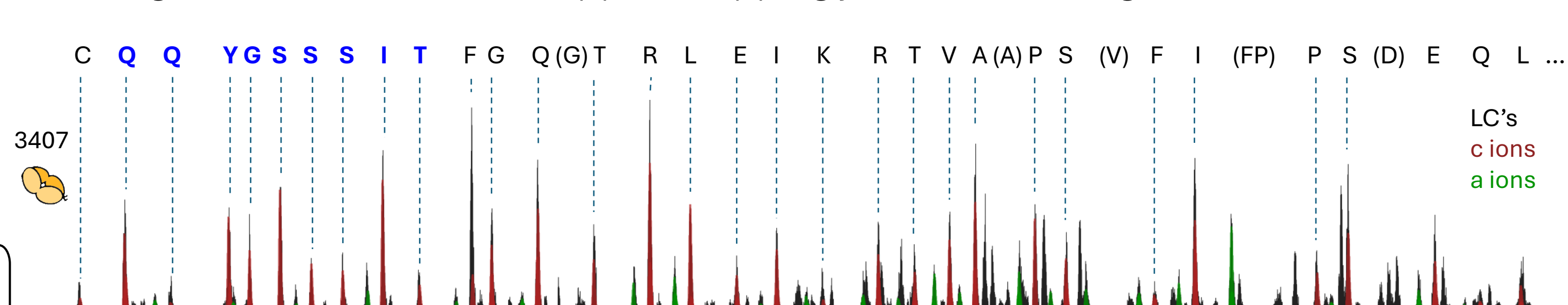
### Charge deconvoluted ECD ion sequence ladder of the LAC49 bsAb F(ab')2 with one O-glycan

The complete ion sequence ladders are explained, unambiguously confirming the CDR3 assignments.



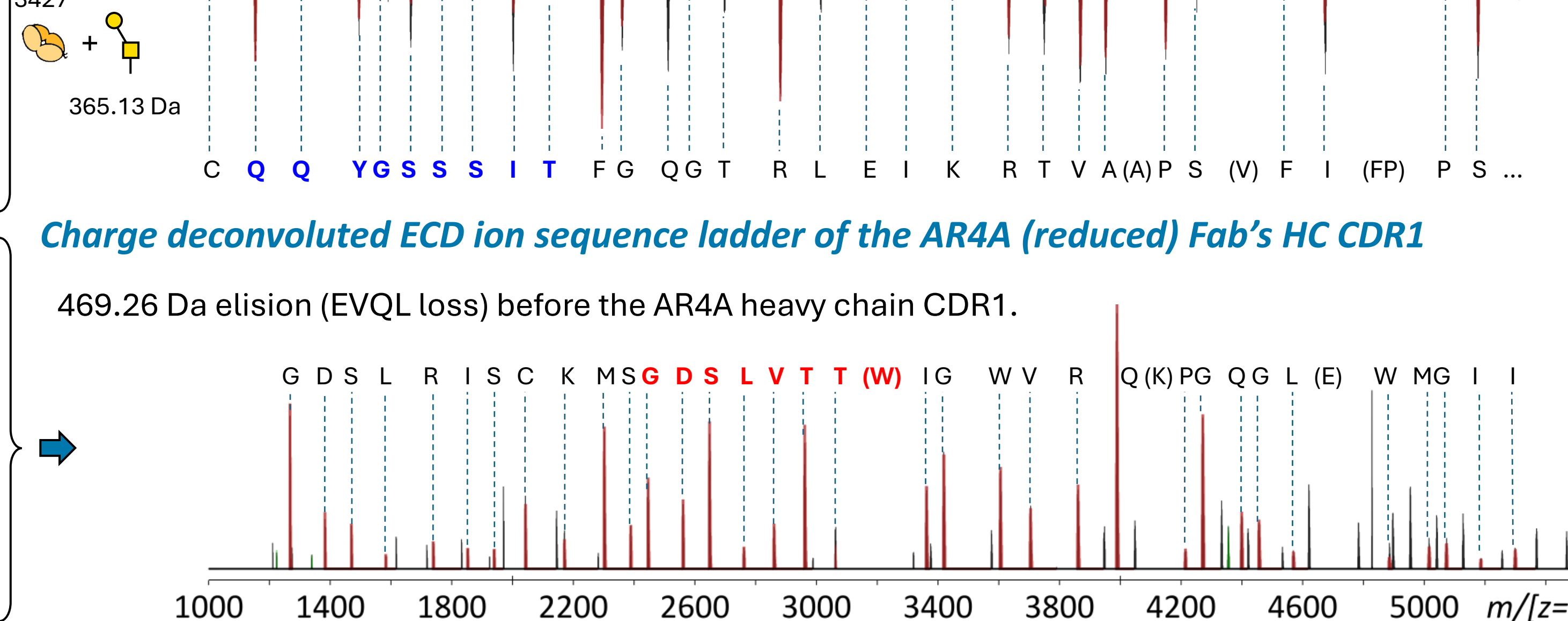
### Charge deconvoluted ECD ion sequence ladder of the AR4A Fab's LC CDR3 w.o./w. 1 O-glycan

Unambiguous localization of the Hex(1)HexNac(1) O-glycan on the AR4A light chain before the CDR3.



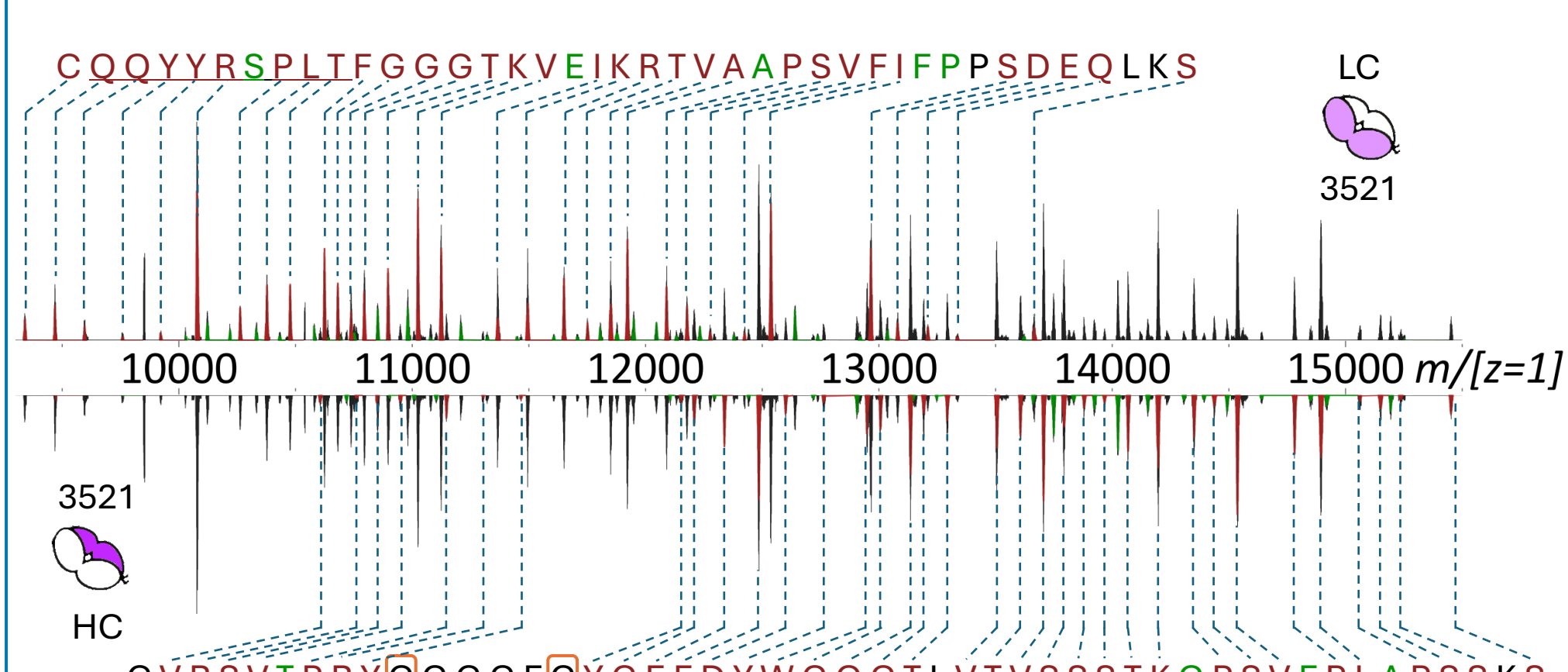
### Charge deconvoluted ECD ion sequence ladder of the AR4A (reduced) Fab's HC CDR1

469.26 Da elision (EVQL loss) before the AR4A heavy chain CDR1.

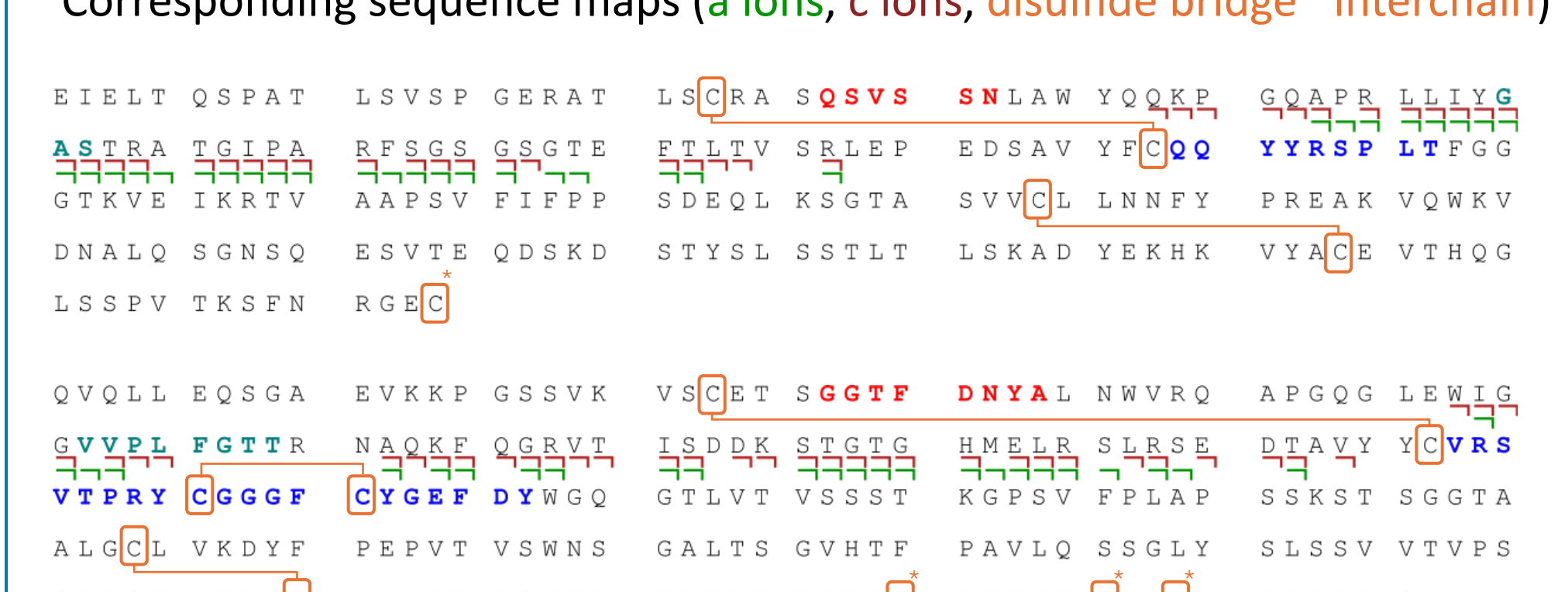


### Easy-to-read ECD fragment ion ladders of LAC49's AR3C Fab

AR3C Fab's LC (top) & HC (flipped) CDR3, FR4, CR fragments' assignments.

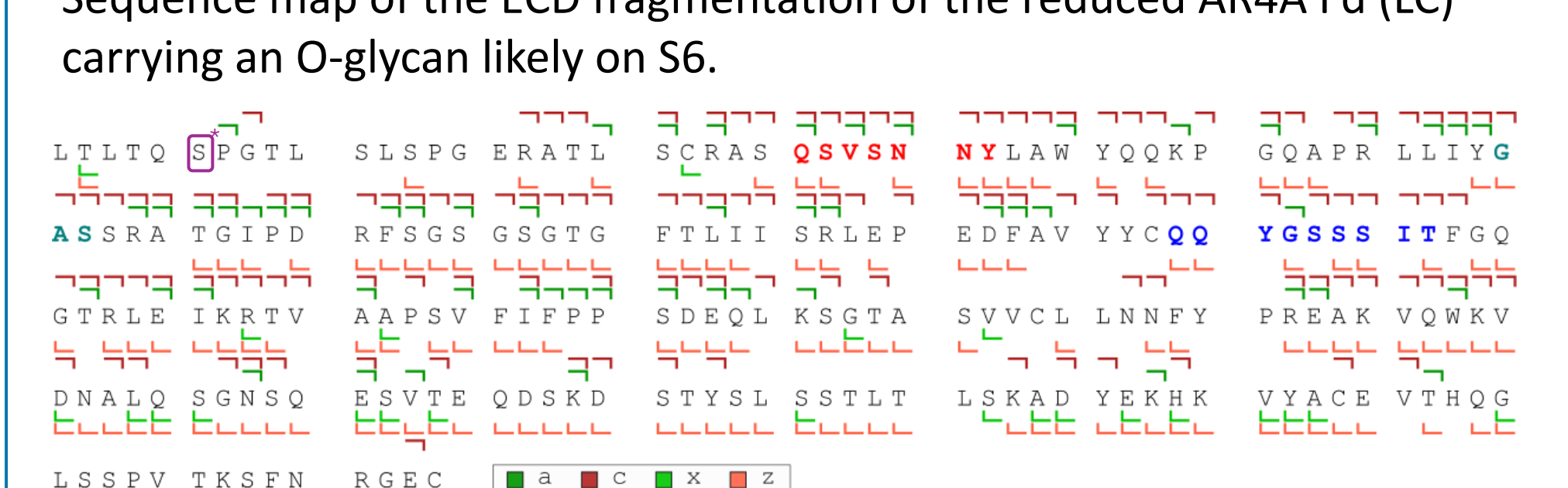


Corresponding sequence maps (a ions, c ions, disulfide bridge \*interchain)



### Easy-to-read ECD fragment ion ladders of LAC49's AR4A Fab

Sequence map of the ECD fragmentation of the reduced AR4A Fd (LC) carrying an O-glycan likely on S6.



## Conclusions

- The timsOmni delivers high quality top-down fragmentation spectra facilitating processing by enabling visual comparison and interpretation.
- Using ECD on the timsOmni, some of the current top-down limitations in terms of sequence coverage for >25kDa proteins can be overcome, e.g. disentanglement of the fragments of a 100 kDa bsAb's proteoform to unambiguously identify and localize posttranslational modifications.
- In short, sample processing with the timsOmni enables characterizing proteoform heterogeneity as well as monitoring modifications and mutations occurring during production and storage.

## Future work

- Use collision-induced unfolding on the timsOmni to populate conformers prior to their fragmentation: MS3 workflow.
- Perform ion mobility separation before mass selection and fragmentation in the Omnitrap to untangle proteoforms.

## Acknowledgments & contact

- The (bispecific) antibodies analyzed here are targeting the hepatitis C virus E1E2 envelope glycoprotein, as described in ref. 1, and provided by Laura Radić.
- Ref. 1. Bispecific antibodies against the hepatitis C virus E1E2 envelope glycoprotein. Radić L. & al. Proc Natl Acad Sci U S A. 2025, doi: 10.1073/pnas.2420402122
- Ref. 2. Selectivity over coverage in de novo sequencing of IgGs. den Boer MA, Greisch JF, Tamara S, Bondt A, Heck AJR. Chem Sci. 2020, doi: 10.1039/d0sc03438j
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