N- and O-Glycosylation in Recombinant SARS-CoV-2 RBD Expression Products Analyzed by Next Generation MALDI Top-Down Sequencing

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Arndt Asperger[1]; Anja Resemann[1]; Waltraut Evers[1]; Niels Goedecke[1]; Ryan Marsico[2]; Detlev Suckau[1]

[1]Bruker Daltonics GmbH & Co. KG, Bremen, Germany; [2]Bruker Scientific LLC, Billerica, MA

Introduction

The receptor binding domain (RBD) of SARS-CoV-2 S-glycoprotein plays a key role in the interaction with the ACE2 receptor on host cells and is, therefore, of interest for the development of rapid COVID19 antigen tests. As protein modifications are affecting protein function, RBD expression products need to be characterized in depth with regard to their N- and O-glycosylation profile.

We describe here a novel method for rapid Top-Down analysis of N- and O-glycosylation in recombinant SARS-CoV-2 RBDs utilizing Next Generation MALDI Top-Down Sequencing (Next Gen MALDI-TDS). Next Gen MALDI-TDS combines MALDI In-Source Decay (ISD) with ultrafast separation by Trapped Ion Mobility Spectrometry (TIMS), and high-resolution, accurate time-of-flight mass analysis (TOF-MS), delivering information simultaneously regarding primary sequence, terminal status and near-terminal modifications of intact proteins.

Methods

SARS-CoV-2 RBDs (with a C-terminal His6-tag added) were expressed in CHO and HEK293 cells (InVivo Biotech Services). N-linked glycans were released with PNGase F (Promega). Further deglycosylating enzymes, SialEXO and OglyZOR (Genovis), were applied for removal of sialic acids and core-1 O-glycans, resp..

Next Generation MALDI Top-Down Sequencing data were acquired on a Bruker timsTOF flex instrument equipped with ESI/MALDI dual ion source from a sample amount of approx. 20-40 pmol RBD using sDHB as MALDI matrix. Data were analyzed in DataAnalysis, BioPharma Compass 2021 and Biotools software (Bruker). For more detailed information on sample prep protocols refer to [1]. Data acquisition methods are described at greater detail in [1,2].

Results

O-Glycosylation (Figures 1 & 2)

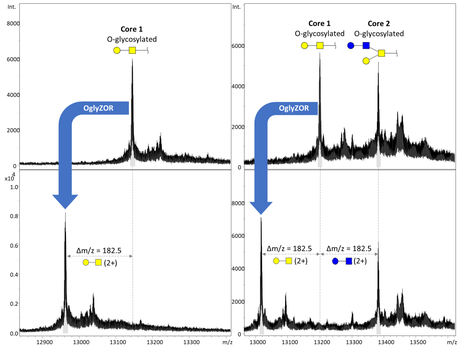

Figure 1: timsTOF fleX MALDI-MS intact mass analysis of PNGaseF/SialEXO treated SARS-CoV-2 RBD expression products. Data obtained before (***top***) and after (***bottom***) OglyZOR treatment indicate presence of a single O-glycosylation in both CHO- and HEK-RBDs. In CHO-RBD (***left***), core-1 was detected as the only O-glycan type, whereas data obtained from HEK-RBD (***right***) indicate presence of core-1 and core-2 O-glycans.

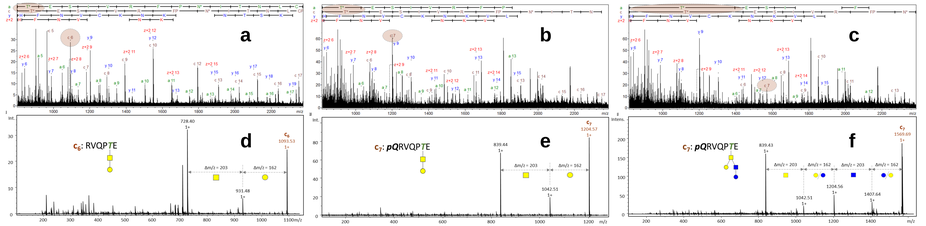

Figure 2: timsTOF fleX MALDI-ISD-MS spectra of PNGaseF/SialEXO treated CHO- (***a***) and HEK-RBD (***b:*** matched against core-1 modified sequence; ***c:*** matched against core-2 modified sequence) provide evidence for active O-glycosylation sites **Thr-5** (CHO) and **Thr-6** (HEK), resp.. Diagnostic glycan fragments observed in MALDI-TIMS-MS/MS spectra of N-terminal ISD fragments c_6 (CHO; ***d***) and c_7 (HEK; ***e-f***), resp., further confirm **Thr-5** (CHO) / **Thr-6** (HEK) as the active O-glycosylation site. **Ser-7** (CHO) / **Ser-8** (HEK) are not glycosylated.

N-Glycosylation (Figure 3)

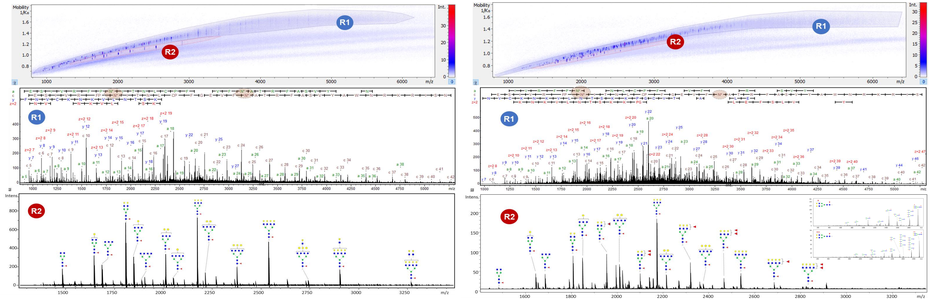

Figure 3: timsTOF fleX MALDI-ISD-TIMS-MS analysis of PNGaseF/SialEXO treated CHO- (***left***) and HEK-RBD (***right***): TIMS is capable of separating released N-glycans (R2) from the complex cloud of protein backbone ISD fragments (R1) enabling dissection of a complete N-glycan fingerprint for compositional profiling. As a unique feature when compared to CHO-RBD, the N-glycan profile in HEK-RBD comprises a significant portion of multiply fucosylated species. Example MALDI-TIMS-MS/MS spectra confirming the identity of Hex(5)HexNAc(4)dHex(2-3) are given in the inset in the bottom-right panel. At the same time, N-glycosylation sites are confirmed by ISD fragments matching aspartic acid residues resulting from deamidation induced by PNGase F digestion.

References

[1] C. Gstöttner, T. Zhang, A. Resemann, S. Ruben, S. Pengelley, D. Suckau, T. Welsink, M. Wuhrer, E. Domínguez-Vega. Anal. Chem. 2021, 93, 17, 6839–6847

[2] A. Asperger, A. Resemann, W. Evers, N. Goedecke, D. Suckau. Bruker Application Note LCMS-186, *www.bruker.com*

Conclusions

- **Next Gen MALDI-TDS** combines MALDI-ISD with TIMS separation and high-resolution TOFMS; it represents a novel, uniquely efficient method for characterization of N- and O-glycosylation in biopharmaceuticals as demonstrated for SARS-CoV-2 S-glycoprotein RBD expression products.
- **Number of O-glycosylation sites and O-glycan profile:** Intact accurate mass analysis revealed the presence of a single O-glycosylation in both CHO- and HEK-RBDs. Core-1 was found in CHO-RBD, core-1 and core-2 O-glycans in HEK-RBD. (**Fig. 1**)
- **Localization of O-glycosylation sites:** MALDI-ISD-MS analysis with subsequent TIMS-MS/MS of selected N-terminal ISD fragments provided evidence for Thr-5 in CHO and Thr-6 in HEK as the sole O-glycosylation site. (**Fig. 2**)
- **N-glycan profile:** PNGaseF-released N-glycans separate on the MALDI-ISD-TIMS-MS heatmap from ISD protein fragments allowing for dissection of high-quality N-glycan profiles. (**Fig. 3**)
- **N-glycosylation sites** were identified based on assignment of N-terminal MALDI-ISD fragments matching deamidated asparagine residues resulting from PNGase F treatment. (**Fig. 3**)

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