Supporting Information

Glycan and protein analysis of glycoengineered bacterial *E. coli* vaccines by MALDI-in-source decay FT-ICR mass spectrometry

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Table of Content

| Name     | Description                                                                 | Page # |
|----------|------------------------------------------------------------------------------|--------|
| Figure S1 | MALDI-ISD FT-ICR mass spectra of glycoconjugate EcoO25B acquired in positive and negative ion modes | S3     |
| Figure S2 | Enlargements of the MALDI-ISD FT-ICR mass spectrum of glycoconjugate EcoO25B depicted in Figure S1A | S4     |
| Figure S3 | Examples of fragment ions detected in positive and negative ion modes       | S5     |
| Figure S4 | Enlargements of negative mode MALDI-ISD FT-ICR mass spectra of the glycoconjugates EcoO2, EcoO6A, and EcoO25B | S7     |
| Figure S5 | Enlargements of negative mode MALDI-ISD FT-ICR mass spectra of the glycoconjugates EcoO2, EcoO6A, and EcoO25B | S7     |
| Figure S6 | Schematic representation of the standardized nomenclature of fragment ions of carbohydrates | S8     |
| Figure S7 | Enlargement of the MALDI-ISD FT-ICR mass spectra of the glycoconjugates EcoO2, EcoO6A, and EcoO25B in the m/z-range 3000-8000 | S9     |
| Figure S8 | Enlargement of the MALDI-ISD FT-ICR mass spectrum of the glycoconjugate EcoO6A in the m/z-range 4000-12000 | S10    |
| Figure S9 | Enlargement of the MALDI-ISD FT-ICR mass spectrum of the glycoconjugate EcoO6A in the m/z-range between 1RU and 2RU | S11    |
| Figure S10| Enlargement of the MALDI-ISD FT-ICR mass spectrum of the glycoconjugate EcoO25B in the m/z-range between 1RU and 2RU | S12    |
Figure S1. Examples of MALDI-ISD FT-ICR mass spectra acquired in (A) positive and (B) negative ion modes after desalting of the glycoconjugate EcoO25B. Such spectra were visually investigated and the signals matching the theoretical m/z-values of ISD fragment ions of EPA protein were assigned (see Figure S2). The lists of the assigned fragment ions are reported in Table S4. Enlargements of the spectrum depicted in A are reported in Figures S2. The spectrum depicted in B is characterized by the presence of intense MALDI ion clusters that are typically detected below m/z 1000. Ultrahigh-resolution measurements allowed for confident assignment in this region.
Figure S2. Enlargements of the MALDI-ISD FT-ICR mass spectrum of glycoconjugate EcoO25B depicted in Figure S1A. Detected $c$-, $y$- and $z$'-type fragment ions were compared with theoretical values and assigned within a mass measurement error of 15 ppm. The assigned fragments are reported in Table S4. * indicates $z'$+1,5-DAN ions. Blue circle indicates $w$-type ions.
Figure S2. Continued
Figure S3. Examples of fragment ions detected in positive and negative ion modes. As previously shown (Nicolardi et al., Anal. Chem. 2020, 92, 18, 12429–12436), negative mode MALDI-ISD FT-ICR MS can provide a higher signal intensity of fragment ions in the m/z-region of the MALDI matrix, i.e. typically below m/z 1000.

Figure S4. Enlargements of negative mode MALDI-ISD FT-ICR mass spectra of the glycoconjugates EcoO2, EcoO6A, and EcoO25B. The c’7 fragment ion, generated from the cleavage of the peptide bond between Gln7 and the potentially glycosylated Asn8, was detected in all mass spectra.
Figure S5. Enlargements of negative mode MALDI-ISD FT-ICR mass spectra of the glycoconjugates EcoO2, EcoO6A, and EcoO25B. The c'9 fragment ion included the potentially glycosylated Asn8. This fragment ion, as well as larger c'-type ions, was not detected in the mass spectrum of EcoO6A indicating a high glycosylation site occupancy at Asn8.
Figure S6. Schematic representation of the nomenclature of fragment ions of carbohydrates according to Domon and Costello (Domon, B.; Costello, C. E., Glycoconj. J. 1988, 5 (4), 397-409).
Figure S7. Enlargement of the MALDI-ISD FT-ICR mass spectra of the glycoconjugates EcoO2, EcoO6A, and EcoO25B in the \( m/z \)-range 3000-8000. Sodiated B ions corresponding to up to 9 repeating units were detected in this \( m/z \)-range. The mass spectrum of EcoO25B showed a higher complexity due to the presence of fragment ions generated from the partial loss of acetylation and C-type fragmentation. Not annotated peaks are fragment ions generated from the cleavage of one or more different glycosidic bonds (See Figures 4 and S9-S10).
Figure S8. Enlargement of the MALDI-ISD FT-ICR mass spectrum of the glycoconjugate EcoO6A in the 
$m/z$-range 4000-12000. Sodiated B ions corresponding to up to 11 repeating units were detected in 
this $m/z$-range. Not annotated peaks are B (and C) ions generated from the cleavage of one or more 
different glycosidic bonds (see Figures 3 and S9-S10).
Figure S9. Enlargement of the MALDI-ISD FT-ICR mass spectrum of the glycoconjugate EcoO6A (see Figure 3) in the $m/z$-range between sodiated B-ions corresponding to 1RU and 2RU. The cleavage of one or more glycosidic bonds led to the formation of the fragment ions highlighted in red. Different isomeric fragment ions may exist for each assigned peak.
Figure S10. Enlargement of the MALDI-ISD FT-ICR mass spectrum of the glycoconjugate EcoO25B (see Figure 3) in the m/z-range between sodiated B-ions corresponding to 1RU and 2RU. The cleavage of one or more glycosidic bonds led to the formation of the fragment ions highlighted in red. Different isomeric fragment ions may exist for each assigned peak. * indicates fragment ions that were generated from repeat unit variants lacking one (or more) acetyl groups.