Supplementary Materials for

Site-specific glycan analysis of the SARS-CoV-2 spike

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Published 4 May 2020 on Science First Release
DOI: 10.1126/science.abb9983

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Materials and Methods

Protein expression and purification

To express the prefusion S ectodomain, a gene encoding residues 1–1208 of SARS-CoV-2 S (GenBank: MN908947) with proline substitutions at residues 986 and 987, a “GSAS” substitution at the furin cleavage site (residues 682–685), a C-terminal T4 fibritin trimerization motif, an HRV3C protease cleavage site, a TwinStrepTag and an 8XHisTag was synthesized and cloned into the mammalian expression vector pαH. This expression vector was used to transiently transfect FreeStyle293F cells (Thermo Fisher) using polyethylenimine. Protein was purified from filtered cell supernatants using StrepTactin resin (IBA) or nickel-affinity chromatography before being subjected to additional purification by size-exclusion chromatography using a Superose 6 10/300 column (GE Healthcare) in 2 mM Tris pH 8.0, 200 mM NaCl and 0.02% NaN₃.

Negative-stain electron microscopy and 2D class averaging

Purified SARS-CoV-2 spike was diluted to a concentration of 0.04 mg/mL using 2 mM Tris pH 8.0, 200 mM NaCl and 0.02% NaN₃ before being applied to a plasma cleaned CF400-Cu grid (Electron Microscopy Sciences). Protein was then stained using methylamine tungstate (Nanoprobes) before being allowed to dry at room temperature for 15 minutes. This grid was imaged in a Talos TEM (Thermo Fisher Scientific) equipped with a Ceta 16M detector. Micrographs were collected using TIA v4.14 software at a nominal magnification of 92,000×, corresponding to a calibrated pixel size of 1.63 Å/pix. CTF estimation, particle picking and 2D class averaging were performed using cisTEM (38).

Glycopeptide analysis by mass spectrometry

Three 30 μg aliquots of SARS-CoV-2 S protein, from three biological replicates, were denatured for 1h in 50 mM Tris/HCl, pH 8.0 containing 6 M of urea and 5 mM dithiothreitol (DTT). Next, the S protein were reduced and alkylated by adding 20 mM iodoacetamide (IAA) and incubated for 1h in the dark, followed by a 1h incubation with 20 mM DTT to eliminate residual IAA. The alkylated Env proteins were buffer-exchanged into 50 mM Tris/HCl, pH 8.0 using Vivaspin columns (3 kDa) and digested separately overnight using trypsin chymotrypsin or alpha lytic protease (Mass Spectrometry Grade, Promega) at a ratio of 1:30 (w/w). The next day, the peptides were dried and extracted using C18 Zip-tip (MerckMilipore). The peptides were dried again, re-suspended in 0.1% formic acid and analyzed by nanoLC-ESI MS with an
Easy-nLC 1200 (Thermo Fisher Scientific) system coupled to a Fusion mass spectrometer (Thermo Fisher Scientific) using higher energy collision-induced dissociation (HCD) fragmentation. Peptides were separated using an EasySpray PepMap RSLC C18 column (75 µm × 75 cm). A trapping column (PepMap 100 C18 3µM 75µM x 2cm) was used in line with the LC prior to separation with the analytical column. The LC conditions were as follows: 275 minute linear gradient consisting of 0-32% acetonitrile in 0.1% formic acid over 240 minutes followed by 35 minutes of 80% acetonitrile in 0.1% formic acid. The flow rate was set to 200 nL/min. The spray voltage was set to 2.7 kV and the temperature of the heated capillary was set to 40 °C. The ion transfer tube temperature was set to 275 °C. The scan range was 400−1600 m/z. The HCD collision energy was set to 50%, appropriate for fragmentation of glycopeptide ions. Precursor and fragment detection were performed using an Orbitrap at a resolution MS1=100,000. MS2= 30,000. The AGC target for MS1=4e5 and MS2=5e4 and injection time: MS1=50ms MS2=54ms. Glycopeptide fragmentation data were extracted from the raw file using Byonic™ (Version 3.5) and Byologic™ software (Version 3.5; Protein Metrics Inc.). The glycopeptide fragmentation data were evaluated manually for each glycopeptide; the peptide was scored as true-positive when the correct b and y fragment ions were observed along with oxonium ions corresponding to the glycan identified. The MS data was searched using the Protein Metrics N-glycan library. The relative amounts of each glycan at each site as well as the unoccupied proportion were determined by comparing the extracted chromatographic areas for different glycotypes with an identical peptide sequence. All charge states for a single glycopeptide were summed. The precursor mass tolerance was set at 4 ppm and 10 ppm for fragments. A 1% false discovery rate (FDR) was applied. The relative amounts of each glycan at each site as well as the unoccupied proportion were determined by comparing the extracted ion chromatographic areas for different glycopeptides with an identical peptide sequence. Glycans were categorized according to the composition detected. HexNAc(2)Hex(9–5) was classified as M9 to M5. HexNAc(3)Hex(5–6)X was classified as Hybrid with HexNAc(3)Fuc(1)X classified as Fhybrid. Complex-type glycans were classified according to the number of processed antenna and fucosylation. If all of the following compositions have a fucose they are assigned into the FA categories. HexNAc(3)Hex(3-4)X is assigned as A1, HexNAc(4)X is A2/A1B, HexNAc(5)X is A3/A2B, and HexNAc(6)X is A4/A3B. As this fragmentation method does not provide linkage information compositional isomers are group, so for example a triantennary glycan contains HexNAc 5 but so does a biantennary glycans with a bisect. Any glycan
containing at least one sialic acid was counted as sialylated. The quantifications of glycan compositions were represented as the mean of three biological replicates +/- standard error of the mean.

For O-linked glycan analysis, trypsin and alpha lytic protease-generated glycopeptides were treated with PNGase F prior to analysis to remove N-linked glycans. This was performed on a single biological replicate using an HCD energy of 27%. The MS data was searched using the Protein Metrics 70 common O-linked glycan library.

**Model construction**

Structural models of N-linked glycan presentation on SARS-CoV-2 were created using electron microscopy structures (PDB ID: 6VSB) along with complex-, hybrid-, and oligomannose-type N-linked glycans (PDB ID 4BYH, 4B7I, and 2WAH). A representative glycan presented at each site was modelled on to the N-linked carbohydrate attachment sites in Coot (39).
Supplementary Figure S1. Size-exclusion chromatogram of the affinity purified SARS-CoV-2 S protein. The elution volume of 670 kDa corresponding to the trimeric mass of the protein is shown.
### Supplementary Table S1. Glycoform abundances observed across SARS CoV-2 S protein.

The relative abundances of all N-linked glycans detected at each site is displayed. The displayed value is the mean percentage abundance of each glycan detected across three biological replicates. See also Figure 2.

| Site       | N-linked Glycans | A | B | C | D | E | F | G | H | I | J | K | L |
|------------|------------------|---|---|---|---|---|---|---|---|---|---|---|---|
| 1          | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2          | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 3          | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 4          | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 5          | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 6          | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 7          | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 8          | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 9          | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 10         | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 11         | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 12         | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 13         | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 14         | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 15         | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 16         | 0.0              | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

See also Figure 2.
Supplementary Figure S2. Extended site-specific N-linked glycosylation of SARS-CoV-2 S glycoprotein. All glycan compositions detected across every N-linked glycan site are listed and numbered along the x-axis. The corresponding glycan compositions can be found in supplementary table S1. The bar graphs represent the mean abundance of each glycan of three biological repeats (+/- SEM) with oligomannose-type glycan series (green), hybrid glycans (dashed pink), and complex glycans (pink). Glycan sites are colored according to oligomannose-type glycan content with the glycan sites labelled in green (80–100%), orange (30–79%) and pink (0–29%). See also Figure 2.
Supplementary Table S2. Glycoform abundances observed across SARS CoV-2 S protein. The upper table shows the categorized glycan compositions at each N-linked glycan site with the reported value the mean of three biological replicates. The global averages are shown in the right-hand table. The lower table further categorizes the glycan compositions into oligomannose-, hybrid-, and complex-type as well as the percentage of glycan compositions containing at least one fucose or one sialic acid residue. See also Figure 2.

| N-Linked Site | M9 | M8 | M7 | M6 | M5 | Hybrid | FlyHybrid | At1 | FA1 | A2A1B | FA2FA1B | FA3/A2B | FA3/FA2B | A4/A3B | FA4/F4AB | Unoccupied |
|---------------|----|----|----|----|----|--------|----------|-----|-----|-------|---------|---------|---------|-------|----------|------------|
| Value (%)     |    |    |    |    |    |        |          |     |     |       |         |         |         |       |          |            |
| Mesnose       | 43 | 46 | 46 | 33 | 38 | 22     | 22       | 22  | 22  | 22    | 22      | 22      | 22      | 22    | 22       | 22          |
| Hybrid        | 2  | 0  | 19 | 1  | 5  | 1      | 0        | 0   | 0   | 0     | 1       | 0       | 0       | 0     | 0         | 0           |
| Complex       | 84 | 22 | 96 | 25 | 91 | 95     | 92       | 92  | 92  | 92    | 96      | 96      | 82      | 92    | 92        | 92          |
| Unoccupied    | 1  | 0  | 1  | 0  | 0  | 0      | 0        | 0   | 0   | 0     | 0       | 0       | 0       | 0     | 0         | 0           |
| Fucosylation  | 95 | 6  | 77 | 18 | 91 | 63     | 54       | 54  | 54  | 54    | 54      | 54      | 54      | 54    | 54        | 54          |
| Sialylation   | 20 | 4  | 18 | 5  | 33 | 18     | 12       | 12  | 12  | 12    | 12      | 12      | 12      | 12    | 12        | 12          |

| Total         |    |    |    |    |    |        |          |     |     |       |         |         |         |       |          |            |
| Value (%)     |    |    |    |    |    |        |          |     |     |       |         |         |         |       |          |            |
| Mesnose       | 98 | 98 | 98 | 98 | 98 | 98     | 98       | 98  | 98  | 98    | 98      | 98      | 98      | 98    | 98        | 98          |
| Hybrid        | 2  | 2  | 2  | 2  | 2  | 2      | 2        | 2   | 2   | 2     | 2       | 2       | 2       | 2     | 2         | 2           |
| Complex       | 84 | 22 | 96 | 25 | 91 | 95     | 92       | 92  | 92  | 92    | 96      | 96      | 82      | 92    | 92        | 92          |
| Unoccupied    | 1  | 1  | 1  | 1  | 1  | 1      | 1        | 1   | 1   | 1     | 1       | 1       | 1       | 1     | 1         | 1           |
| Fucosylation  | 95 | 6  | 77 | 18 | 91 | 63     | 54       | 54  | 54  | 54    | 54      | 54      | 54      | 54    | 54        | 54          |
| Sialylation   | 20 | 4  | 18 | 5  | 33 | 18     | 12       | 12  | 12  | 12    | 12      | 12      | 12      | 12    | 12        | 12          |
Supplementary Figure S3. Glycosylated model of SARS-CoV-2 S glycoprotein highlighting different glycan modifications. The experimentally determined quantities of mannosylation, fucosylation and sialylation presented in Supplementary Table S2 were used to color the glycosylated model of SARS-CoV2 S protein presented in Figure 3. Glycans are highlighted according to mannosylation (A), fucosylation (B) and sialylation (C) levels as denoted in the keys. S1 and S2 subunits are colored light grey and dark grey, respectively.
**Supplementary Figure S4. Detection of low levels of mucin-type O-linked glycosylation at T323/S325 of SARS-CoV-2 S.** O-linked glycan compositions were observed at T323/S325. This analysis was performed on a single biological replicate. For each peptide/glycopeptide detected the extracted ion chromatogram (XIC) (A), isotope distribution (B), and fragmentation spectrum (C) is shown. The monoisotopic peak m/z is labelled in (B). Fragment ions are colored blue and red for b- and y-ions, respectively. Oxonium ions are colored in green.

**Data S1** (separate Excel file): Assigned peptide/glycopeptide list.
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