Supporting Information

Linkage-specific sialic acid derivatization for MALDI-TOF-MS profiling of IgG glycopeptides

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S-1. Samples

S-1.1. Chemicals, reagents, enzymes

The ultrapure deionized water (MQ) used in this study was generated from a Q-Gard 2 system (Millipore, Amsterdam, Netherlands), maintained at ≥18 MΩ. Ethanol (EtOH), methanol (MeOH), trifluoroacetic acid (TFA), sodium dodecyl sulfate (SDS), glacial acetic acid, disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), potassium dihydrogen phosphate (KH_2PO_4) and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). TPCK treated trypsin (from bovine pancreas), 2-aminobenzamide (2-AB), 2-picoline borane (2-PB), HOBt hydrate, dimethyl sulfoxide (DMSO), 50% sodium hydroxide (NaOH), 40% dimethylamine in water, super-DHB, Nonidet P-40 substitute (NP-40), sodium bicarbonate and formic acid (FA) were all purchased from Sigma-Aldrich (Steinheim, Germany) and EDC hydrochloride from Fluorochem (Hadfield, UK). Peptide-*N*-glycosidase F (PNGase F) was acquired from Roche Diagnostics (Mannheim, Germany), CMP-*N*-acetylneuraminic acid (CMP-NANA) sodium salt from Roche Custom Biotech (Mannheim, Germany), 2,5-dihydroxybenzoic acid (2,5-DHB) from Bruker Daltonics (Bremen, Germany), 4-chloro-α-cyanocinnamic acid (Cl-CCA) from Bionet Research (Camelford, Cornwall, UK) and HPLC SupraGradient acetonitrile (ACN) from Biosolve (Valkenswaard, Netherlands). 10x Phosphate-buffered saline (10x PBS) was made in-house, containing 57 g/L $Na_2HPO_4 \cdot 2H_2O$, 5 g/L KH_2PO_4 and 85 g/L NaCl.

S-1.2. MAb2 production

For mAb2 production, 1 g mAb1 was treated in reaction buffer (10 mM UDP-Gal, 20 mM $MnCl_2$, 100 mM MES pH 6.5) with a total of 92 U β(1-4)-galactosyltransferase (Sigma-Aldrich) for 4 days at 32 °C. After purification by protein A chromatography, 500 mg of the highly galactosylated mAb1 was treated with 50 mg ST6-variant1 (Roche enzyme in development; preferentially yielding monosialylated glycans with α2,6-linkage of the sialic acid) in 25 mL of an aqueous CMP-NANA solution at a concentration of 10 mg/ml for 24 h at 37 °C. Proteins were again purified by protein A chromatography. To further increase the degree of sialylation, 100 mg of the mono-sialylated sample was mixed with 3 mL of the aqueous CMP-NANA solution and 10 mg ST6-Variant2 (Roche, Cat. No. 07012250103; preferentially yielding disialylated glycans) and incubated for 7 h at 37 °C with subsequent protein A purification.

S-1.3. BRAD-3 purification

Ten microliter 0.58 mg/mL BRAD-3 sample was purified using protein G Sepharose beads (GE Healthcare, Uppsala, Sweden). The beads were prepared by resuspending them two times in an equal volume of 1x PBS. Of the prepared beads, 15 µL was loaded on a 96-well filter plate (0.7 mL/well, PE frit, Orochem, Naperville, IL), together with 35 µL PBS. On plate, the beads were washed three times with 200 µL PBS using a vacuum manifold. The sample in 200 µL PBS was added to the beads, which were then incubated on a shaking platform (1000 rpm, Heidolph Titramax 100; Heidolph, Kelheim, Germany) for 1 h at room temperature. The beads with captured IgGs were washed three times with 200 µL PBS and three times with 200 µL MQ by vacuum. For elution, 100 µL 100 mM FA in MQ was added to the beads, which were then incubated for 5 min on the shaking platform (600 rpm). The eluate was collected by centrifuging the filter plate for one minute at 50 *g*. The collected eluate was dried and the proteins were dissolved in 23.2 µL 50 mM sodium bicarbonate (resulting in 0.25 mg/mL). The samples were stored at -20 °C until use.

S-1.4. Plasma IgG isolation

Human donor blood was collected in EDTA tubes and centrifuged for 10 min at 2500 *g*. The top layer (the plasma) was transferred to Eppendorf tubes and stored at -20 °C until use.

Plasma IgG was isolated using protein G Sepharose beads as described above. With the adaptation that 2 µL plasma in 200 µL PBS was added to the beads during sample loading. Furthermore, after drying, the proteins were dissolved in 20 µL 50 mM sodium bicarbonate per well (resulting in approximately 1 mg/mL).

S-2. Preparation of glycans and glycopeptides

S-2.1. Glycan release

Prior to the release, 10 µL sample was denatured by adding 20 µL of 2% SDS and incubating for 10 min at 60 °C. To release the glycans, 20 µL of 2% NP-40 and 0.5 mU PNGase F in 2.5x PBS was added and the samples were incubated overnight at 37 °C.

S-2.2. Linkage specific sialic acid derivatization

For the linkage-specific sialic acid derivatization, 1 µL of PNGase F-released glycans was added to 20 µL ethylation reagent, consisting of 250 mM EDC and 250 mM HOBt in EtOH. The mixture was incubated for 1 h at 37 °C, after which 20 µL ACN was added and another incubation of 15 min at -20 °C took place.

S-2.3. Cotton HILIC enrichment

Purification of both released glycan and glycopeptide derivatives was performed by micro tip cotton hydrophilic interaction liquid chromatography (HILIC) solid phase extraction (SPE). Prior to purification, the glycopeptide samples were prepared by bringing them to 85% ACN, whereas the ethyl esterified released glycans were extracted directly from the 1:1 ethanol:ACN mixture. The micro tips were handcrafted as follows: 20 µL pipette tips were packed with 3 mm cotton thread (180 µg, Pipoos, Utrecht, Netherlands) by the application of air pressure (50 kPa pressure difference). Prepared micro tips were conditioned by pipetting three times 15 µL MQ, followed by equilibration with three times 15 µL 85% ACN. The samples were loaded by pipetting 20 times up and down in the reaction mixture. Tips with the loaded sample were then washed three times with 15 µL of 85% ACN 1% TFA and three times with 15 µL of 85% ACN. The retained sample was eluted in 10 µL MQ.

S-3 MALDI-TOF(/TOF)-MS

For the MALDI-TOF-MS analysis of both modified and unmodified glycopeptides, 1 µL of the purified sample (19-25 ng of the original protein) was spotted on a polished steel 384 TF MALDI target (Bruker Daltonics) together with 1 µL 5 mg/mL Cl-CCA in 70% ACN. The spots were left to dry by air. For fragmentation, 1 µL of the sample was spotted with 1 µL 5 mg/mL DHB in 50% ACN.

For the analysis of derivatized released glycans, 1 µL of purified sample was spotted on an AnchorChip 800/384 TF MALDI target (Bruker Daltonics), together with 1 µL 5 mg/mL super-DHB with 1 mM NaOH in 50% ACN. The spots were left to dry by air.

MALDI-TOF(/TOF)-MS measurements were performed on a Bruker Daltonics UltraFlextreme. The instrument was equipped with a Smartbeam-II laser and controlled by Flexcontrol 3.4 software. Prior to measuring the samples, the instrument was calibrated with a peptide calibration standard (Bruker Daltonics). MS measurements were performed in reflectron positive ion mode, using an acceleration voltage of 25 kV after 140 ns delayed extraction. A mass window of *m/z* 1000 to *m/z* 5000 was used for all analytes. 200 shots per raster spot were collected in a random walk, to a total of 10,000 laser shots at a laser frequency of 1000 Hz. A high laser power was used to provide high intensity spectra, while monoisotopic resolution was maintained. There was no difference in the laser power used for samples spotted with Cl-CCA or with sDHB. MS/MS spectra were obtained of the ions of interest via laser induced disassociation.

S-4. Data analysis

The spectra shown in the figures were smoothed (Savitzky-Golay), baseline subtracted (Top-hat) and internally recalibrated using flexAnalysis v3.3 (Bruker Daltonics) (**Table S-1**, Supporting Information). Compositions were assigned to the obtained *m/z* values on basis of literature and fragmentation experiments.[1-4] Annotation was carried out according to CFG, using GlycoWorkbench to create the compositional cartoons.[5]

Integration of the spectra was performed using an in-house developed program "MassyTools". In short, raw MALDI-TOF-MS data were internally recalibrated based on the aforementioned calibration list. After calibration, targeted data extraction was performed using a manually determined list of glycan or glycopeptide compositions. For each composition, the area was integrated and the background was corrected based on the local baseline level. Averages and standard deviations were calculated for repeated experiments.

S-5. Analysis of 2-AB labeled standards

Twenty microliter 50 mg/mL 3'-sialyllactose and 6'-sialyllactose standards were labeled with 2-AB by incubating the samples with 20 µL 24 mg/mL 2-AB and 53.5 mg/mL 2-PB in 7.5%/92.5% acetic acid/DMSO for two hours at 60 °C. 4 µL of each sample was brought to 90% ACN and purified by cotton HILIC-SPE, using 90% ACN. Samples were eluted in 10 µL mQ.

One microliter of the sialyllactose standards (10 mg/mL) was derivatized for 3 h at 60 °C, using 20 µL of the optimal dimethylamidation reagent (250 mM EDC, 500 mM HOBt and 250 mM dimethylamine in DMSO). Samples were purified by cotton HILIC-SPE, using 90% ACN and measured by MALDI-TOF-MS with a mass window of *m/z* 500 to *m/z* 1000. Both the protonated and the sodiated signals of the sialyllactose standards were summed for data extraction.

Five picomol of the A3 standard was similarly derivatized by 20 µL dimethylamidation reagent. Samples were purified by cotton HILIC-SPE, using 85% ACN and measured by MALDI-TOF-MS with a mass window of *m/z* 1000 to *m/z* 5000.

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