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SUPPORTING INFORMATION

Isolation of multiantennary *N*-glycans from glycoproteins for hepatocyte specific targeting via the asialoglycoprotein receptor

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Experimental - Supporting Information

Enzymatic digestion

Fetuin A from fetal calf serum was purchased from Sigma-Aldrich (F3004, Lot-No. 109K7425V, Buchs, Switzerland). Fetuin A has an average molecular weight (MW) of 48.4 kDa (full glycoprotein) including a constant protein part (38.4 kDa) and a variable glycosylation pattern (*N*- and *O*-glycosylations). In the SDS-PAGE analysis fetuin A showed a main band of 63.2 kDa corresponding to the full glycoprotein. It has been reported that the molecular mass varies depending on the carbohydrate content. [1] A standard logMW versus Rf blot was used for the molecular weight determination. For the enzymatic digestion Peptide-*N*-Glycosidase F (PNGase F; 36 kDa) and α2-3,6,8 neuraminidase (Sialidase; 43 kDa) (Fig. S3) were used. Enzymes were obtained from New England BioLabs (Ontario, Canada). Fetuin A was dissolved in ddH_2O containing 0.5% SDS and 40 mM DTT. This solution was heated to 94 °C for 10 min in order to denature the glycoprotein. Phosphate buffer (final concentration 50 mM; pH 7.5), 1% NP40 (detergent), PNGase F (5.37 mU/1 mg fetuin A), sialidase (35U/1 mg fetuin A), and ddH_20 were added to a final fetuin A concentration of 10 mg/mL. Enzymatic digest was for 72 h at 37 °C.

Electrophoretic mobility assay

SDS containing polyacrylamide gels (10%) were prepared using a standard protocol. Samples containing the digested glycoprotein were mixed with reducing sample buffer containing SDS and *β*-mercaptoethanol, heated to 94 °C for 5 min, loaded onto the gel and separated using a current of 30 mA. Proteins were stained using Coomassie blue.

RCA aggregation assay

The Ricinus communis agglutinin (RCA) aggregation assay was performed as described previously. [2,3] In brief, the UV absorbance at 450 nm of 1 mg/mL RCA_{120} (Vector Laboratories, Peterborough, UK) in Dulbecco`s phosphate buffered saline pH 7.4 (Sigma-Aldrich) was measured for 2 min to establish a baseline using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, USA). Glycoprotein was added to initiate lectin aggregation. After 6 min, a galactose solution (10 mg/mL) was added to reverse the aggregation process.

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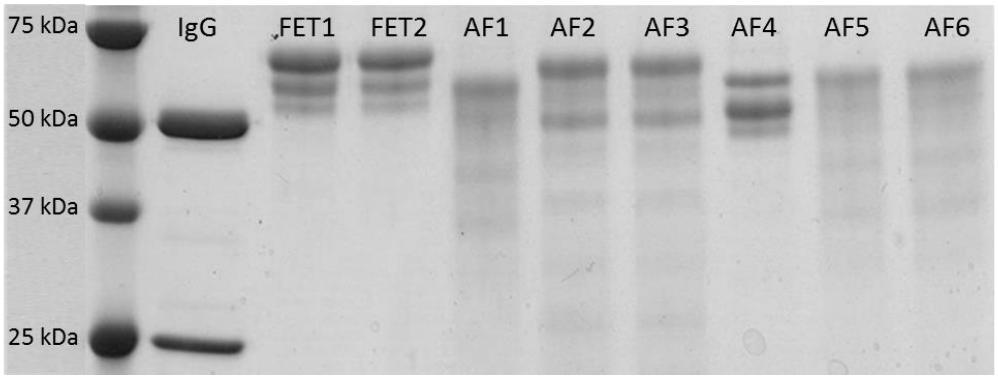

Fig. S1: Analysis of electrophoretic mobility of different fetuin A (FET) and asialofetuin (AF) batches using SDS-PAGE. Proteins were stained using Coomassie blue.

Liquid-liquid extraction

Liquid-liquid extraction of *N*-glycans was performed as described previously with minor modifications. [4] The pH of the digestion mixture was adjusted to 5.5 using an aq.phosphoric acid soln. (8.5%). Acetone was added and the sample was kept for 1 h at -20 °C to precipitate glycans and proteins. The sample was centrifuged for 20 min at 13000 g and 2 °C and the supernatant was discarded. The pellet was resuspended using 1 mL of an aq. methanol soln. (60%) at -20 °C and the sample was left for 1 h at -20 °C to precipitate proteins. Finally, the sample was centrifuged for 5 min at 13000 g and 2 °C and the supernatant containing the *N*-glycans was collected. The methanol extraction procedure was repeated twice.

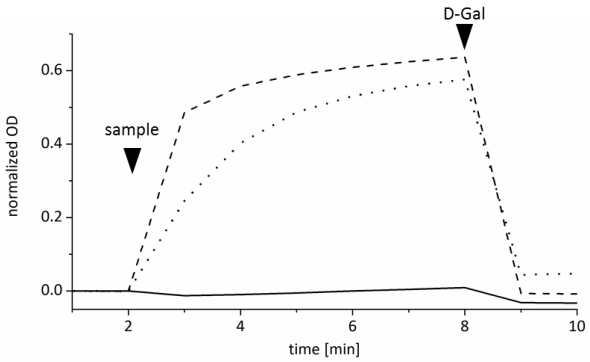

Fig. S2: Ricinus communis agglutinin aggregation assay of fetuin A (solid line), and two different asialofetuin batches (dashed and dotted line). Black triangles: in presence of desialylated glycoprotein (sample), aggregation is induced; competitive de-aggregation by a 100-fold excess of free D-galactose (D-Gal).

Size exclusion chromatography

Isolated *N*-glycans were separated from protein residuals and fractionated using a Superdex 30 Prep Grade column (XK 16/70, GE Healthcare, Glattbrugg, Switzerland) eluting with an aq. phosphate buffer (10 mM, pH 7.4). Collected fractions were dried under nitrogen.

BCA assay

Multiantennary glycan fractions containing terminal sugars with a free and reducing anomeric carbon were identified using a modified bicinchoninic acid (BCA) assay (ThermoFisher Scientific, Zug, Switzerland). [5,6] In brief, samples were mixed with the working reagent (50:1, reagent A:B) in a 1:1 ratio. Samples were heated for 30 min at 65 °C using a water bath, allowed to cool down to room temperature and analysed by UV absorbance measurement at 562 nm.

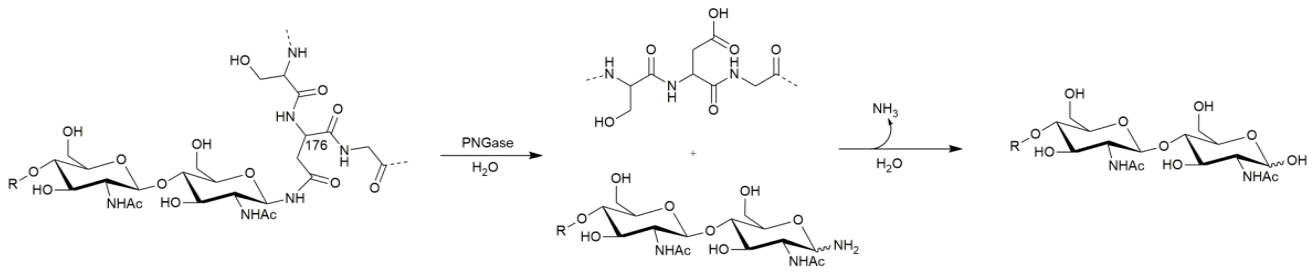

Fig. S3: Enzymatic digestion of bovine fetuin A using Peptide-*N*-Glycosidase F (PNGase) leading to deglycosylation at the representative amino acid position 176. PNGase F selectively cleaves the bond between asparagine and *N*-linked complex carbohydrates. Terminal sialic acid on multiantennary *N*-glycans was cleaved by sialidase.

Bradford assay

Proteins were detected using the Bradford assay (Bio-Rad Laboratories). Samples (160 µl) were mixed with 40 µl dye reagent containing Coomassie blue, methanol, and phosphoric acid. After 10 min incubation at room temperature, the absorbance at 595 nm was determined.

PSAA assay

To detect carbohydrates, the phenol–sulfuric acid assay (PSAA) was used. [7] In brief, the sample was mixed with a threefold volume of concentrated sulfuric acid. Then, an aq. phenol soln. (5%) was added and the mixture was incubated for 5 min at 90 °C. Absorbance was measured at 490 nm.

MALDI-TOF-MS

Fractions containing *N*-glycans were prepared for matrix-assisted laser desorption ionization time of flight mass spectrometer (MALDI-TOF-MS) analysis as follows: The 2,5-dihydroxy benzoic acid (DHB) matrix was spotted onto a MALDI-TOF-MS target plate and allowed to dry. Samples were added to each spot and allowed to dry before analysis. Mass spectra were recorded using a Bruker Ultraflex MALDI-TOF-MS (Bruker, Bremen, Germany). Spectra were analysed using the Bruker Daltonics flex analysis software.

Fluorescent labelling

In order to label isolated *N*-glycans with fluorescein-5-thiosemicarbazide (FTSC; Sigma-Aldrich), a carbohydrate sample (250 µg) was dissolved in 400 µL of a mixture of DMSO and aq. 0.1 M acetic acid soln. (7:3), FTSC (0.25 mg) dissolved in DMF (20 µL) was added and the mixture was stirred for 30 min. To reduce the hydrazone bond and to stabilize the conjugate, an excess of sodium cyanoborhydride was added (500 mM final concentration). The mixture was stirred for 2 h at 70 °C and 700 rpm. Finally, potassium hexacyanoferrate (20 mg) was added to quench the excess of cyanoborhydride and to increase the fluorescence of the fluorophore. The mixture was stirred for 60 min at room temperature. Figure S4 shows the chemical reaction of fluorescent labelling of isolated *N*-glycans. The FTSC-labelled *N*-glycans were purified by gel filtration chromatography (Sephadex G10; GE Healthcare, Glattbrugg, Switzerland; mobile phase of 0.01 M phosphate buffered saline (PBS), pH 7.4).

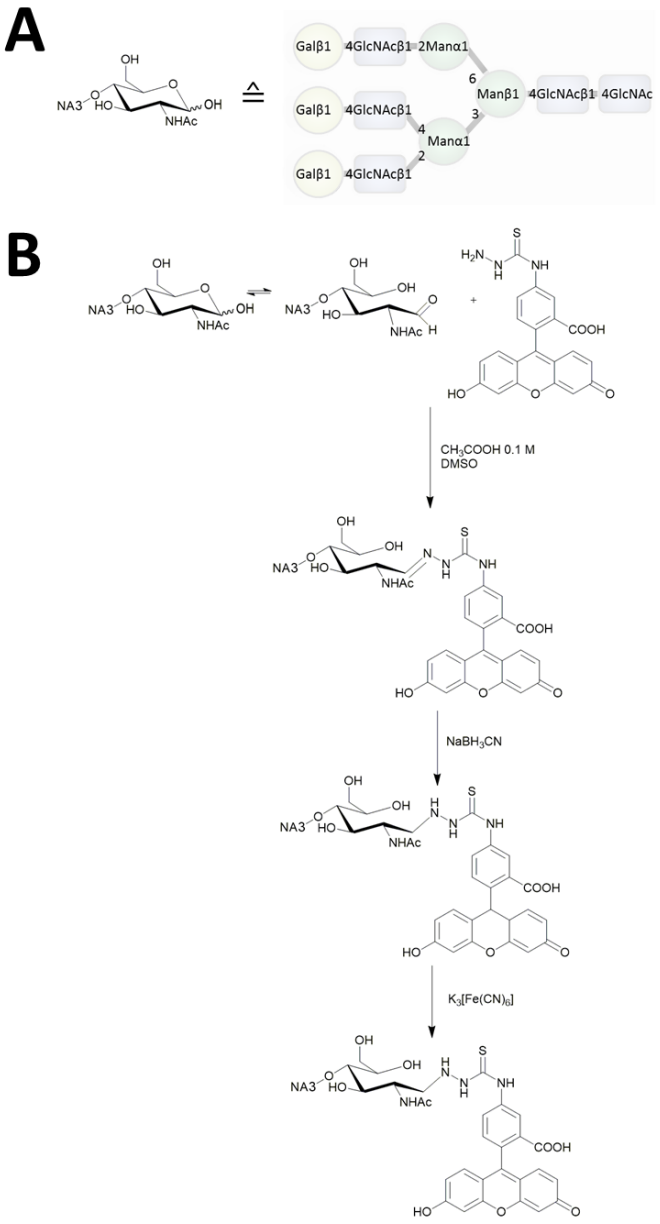

Fig. S4: Fluorescent labelling of complex carbohydrates. (A) Representation of a desialylated triantennary *N*-glycan (NA3) cleaved from asparagine 176 of bovine fetuin A. (B) Chemical reactions for fluorescent labelling of isolated *N*-glycans with fluorescein-5-thiosemicarbazide (FTSC) via reductive amination. Gal: galactose; Man: mannose; GlcNAc: *N*-acetyl glucosamine.

Uptake experiment

Human hepatocellular carcinoma cells (HepG2) were kindly provided by Prof. Dr. Dietrich von Schweinitz (University Hospital Basel, Switzerland). Cells were cultured at 37 °C in a 5% CO_2 atmosphere. All cell culture reagents were purchased from ThermoFisher Scientific (Zug, Switzerland). Dulbecco`s modified Eagle`s culture medium (4.5 g/L glucose) was supplemented with 10% fetal calf serum (FCS), penicillin (100 units/mL) and streptomycin (100 µg/mL). For uptake experiments, HepG2 cells were washed with PBS, detached using accutase and then seeded into 12-well plates at a

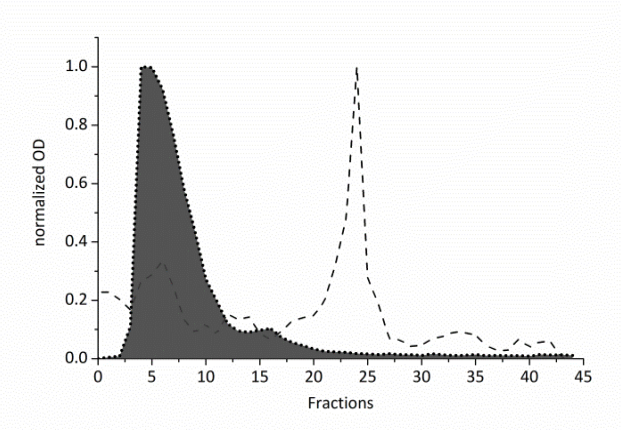

Fig. S5: Size exclusion chromatography fractions of enzymatically digested fetuin A were analysed using different colorimetric assays. The Bradford assay (dotted line, grey) represents the protein residues. The phenol–sulfuric acid assay (dashed line, white) detects carbohydrates. A baseline separation of proteins and free glycans was achieved.

density of 10^5 cells/well. They were allowed to adhere for 24 h. For confocal laser scanning microscopy experiments, cells were cultured on poly-D-lysine coated glass cover slips. Uptake experiments were performed as described previously. [8] In brief, HepG2 cells were incubated with 300 nM fluorescent glycans for 30 min at 37 °C in DMEM without FCS. As a control, cells were co-incubated with asialofetuin (30 µM) or GalNAc (300 µM) or incubations were carried out at 4 °C. Confocal laser scanning microscopy and flow cytometry were used for qualitative and quantitative analysis of uptake, respectively.

Confocal laser scanning microscopy analysis

Cell nuclei were counterstained using 1.0 µg/mL Hoechst 33342 (Sigma-Aldrich). Cells were washed with PBS and then embedded using ProLong Gold antifading reagent (Invitrogen Life Technologies, Zug, Switzerland). Confocal laser scanning microscopy analysis was performed using an Olympus FV-1000 inverted microscope (Olympus Ltd., Tokyo, Japan), equipped with a 60x PlanApo N oil-immersion objective (numerical aperture 1.40). Hoechst 33342 and FTSC-glycans were visualized using excitation at 405 nm and 488 nm and emission at 425-475 nm and 500-600 nm, respectively.

Flow cytometry analysis

For flow cytometry analysis, cells were washed with PBS and detached using 0.25% trypsin/EDTA. Cells were washed twice and then re-suspended in FACS buffer (PBS containing 1% FCS, 0.05% NaN_3, and 2.5 mM EDTA). A FACS Canto II flow cytometer (Becton Dickinson, San Jose, USA) was used for the analysis of 10`000 cells per setting. Cells were excited at 488 nm and the emission was detected in FL1 (505LP - 530/30). To evaluate the uptake rate of FTSC-glycans, Flow Jo VX software (TreeStar, Ashland, OR) was used.

Table S1: Most abundant complex carbohydrates detected after isolation procedure using MALDI-TOF-MS.

Detected Signal $[M+Na]^+$	Attached to	Glycan structure	Reference
1664	ASN-99 and ASN-156	[NA2], e.g. Gal(b1-4)GlcNAc(b1-2)Man(a1-3)[Gal(b1-4)GlcNAc(b1-2)Man(a1-6)]Man(b1-4)GlcNAc(b1-4)GlcNAc	9–11
2029	ASN-99, ASN-156 (major) AND ASN-176	[NA3], e.g. Gal(b1-4)GlcNAc(b1-2)[Gal(b1-4)GlcNAc(b1-4)]Man(a1-3)[Gal(b1-4)GlcNAc(b1-2)Man(a1-6)]Man(b1-4)GlcNAc(b1-4)GlcNAc	9–11
2029	ASN-99, ASN-156 AND ASN-176 (major)	[NA3], e.g. Gal(b1-3)GlcNAc(b1-4)[Gal(b1-4)GlcNAc(b1-2)]Man(a1-3)[Gal(b1-4)GlcNAc(b1-2)Man(a1-6)]Man(b1-4)GlcNAc(b1-4)GlcNAc	9–11

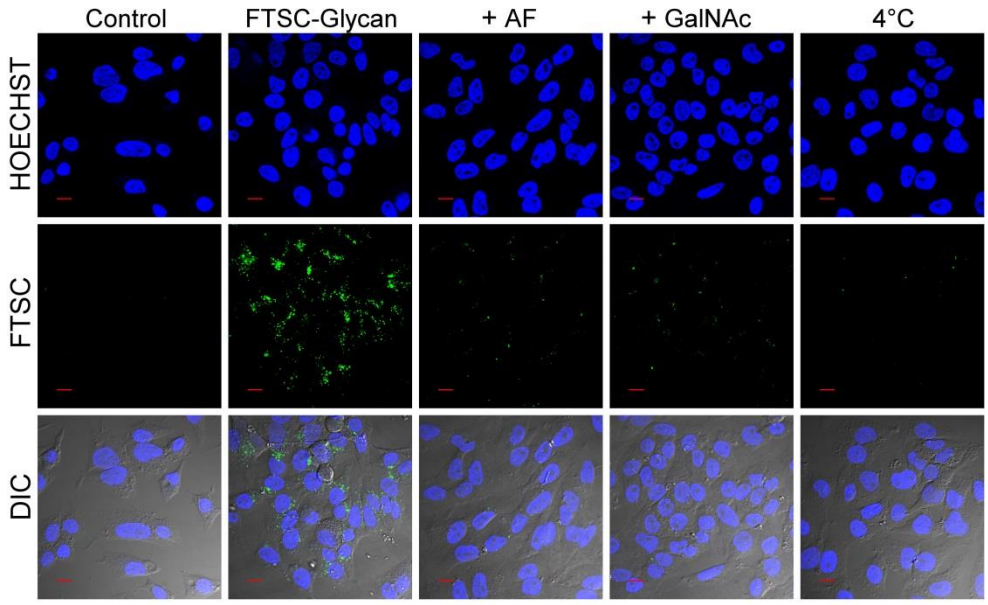

Fig. S6: Confocal laser scanning microscopy analysis of HepG2 cells incubated for 30 min with PBS (control) or FTSC-labelled multivalent N-glycans (green signal). Cellular uptake of N-glycans was competitively inhibited using an excess of asialofetuin (+ AF) or N-acetyl galactosamine (+ GalNAc). Cellular uptake was blocked at low temperature (4 °C). Nuclei are stained using Hoechst 33342 (blue signal). Differential interference contrast (DIC) images are combined with fluorescence signals from cell nuclei and fluorescent N-glycans. Scale bars: 20 µm.

Results - Supporting Information

Electrophoretic mobility batch control

Different batches of fetuin A (FET) and asialofetuin (AF) purchased from Sigma-Aldrich were compared by analysis of their electrophoretic mobility. Figure S1 shows variations in electrophoretic mobility for different asialofetuin batches. Asialofetuin batches AF2 and AF3 show a decreased mobility as compared to other AF batches. These two AF batches have shown a decreased aggregation in the RCA aggregation assay confirming incomplete desialylation of linked glycans (Fig. S2). After a glycan digest using sialidase, the electrophoretic mobility of asialofetuin batches AF2/AF3 was increased and RCA aggregation could be induced. We conclude, that commercial AF (in contrast to fetuin A) shows considerable batch-to-batch differences and that enzymatic treatment (in contrast to chemical processing) of fetuin A leads to better results with respect to completeness of desialylation (AF4). It was therefore decided to use fetuin A as a starting material for further investigations.

RCA aggregation assay batch control

The ricininus communis agglutinin aggregation assay was used as a tool to compare different asialofetuin batches. Figure S2 shows the variation in complexation kinetics of different asialofetuin batches. An excess of galactose (D-Gal) reduces the lectin crosslinking and thus the aggregation.

Analysis of size exclusion chromatography fractions

Size exclusion chromatography was used to separate the different components (i.e. proteins and glycans) of enzymatically digested fetuin. Fractions were analysed

using three different microplate assays (Fig. S5). A Bradford assay was performed to determine the protein content (Fig. S5). As expected, not all proteins have been removed using the liquid-liquid extraction. However, the phenol sulfuric acid assay (PSAA, i.e. total carbohydrate assay) verified, that isolated glycans have successfully been separated from the protein residuals (Fig. S5).

MALDI-TOF-MS

Table S1 summarizes the signals from the MALDI-TOF analysis and published glycosylations reported on unicarbkb.org for bovine fetuin A (Accession number: P12763). [9–11] In total there are 46 associated glycan structures reported with its major glycosylations linked to asparagines at amino acid positions 99, 156, and 176.

Confocal laser scanning microscopy analysis of cellular uptake

The uptake experiments confirm active and specific uptake of fluorescent-tagged, multivalent, isolated *N*-glycans (Fig. S6).

Notes and references - Supporting Information

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