High-Throughput Profiling of Protein N-Glycosylation by MALDI-TOF-MS Employing Linkage-Specific Sialic Acid Esterification

ARTICLE *in* ANALYTICAL CHEMISTRY · MAY 2014

Impact Factor: 5.64 · DOI: 10.1021/ac500335t · Source: PubMed

5 AUTHORS, INCLUDING:

Karli Robert Reiding
Leiden University Medical Centre
10 PUBLICATIONS **54** CITATIONS
SEE PROFILE

Dennis M Kuijper
VU University Amsterdam
1 PUBLICATION **25** CITATIONS
SEE PROFILE

Manfred Wuhrer
Leiden University Medical Centre
195 PUBLICATIONS **5,320** CITATIONS
SEE PROFILE

analytical chemistry

Article

pubs.acs.org/ac

High-Throughput Profiling of Protein N-Glycosylation by MALDI-TOF-MS Employing Linkage-Specific Sialic Acid Esterification

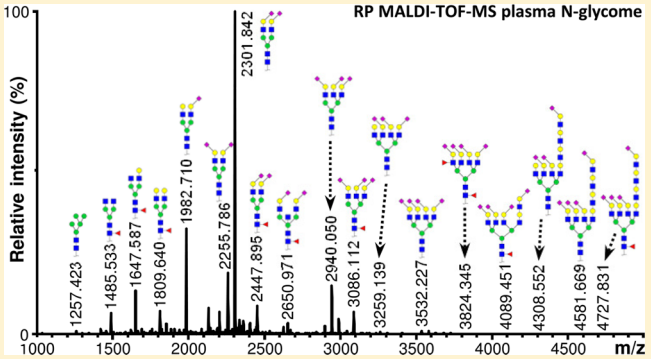
Karli R. Reiding,[†] Dennis Blank,[†] Dennis M. Kuijper,[†,‡] André M. Deelder,[†] and Manfred Wuhrer*[,†,‡]

[†]Center for Proteomics and Metabolomics, Leiden University Medical Center, 2300 RC Leiden, The Netherlands

[‡]Division of BioAnalytical Chemistry, VU University Amsterdam, 1081 HV Amsterdam, The Netherlands

Supporting Information

ABSTRACT: Protein glycosylation is an important post-translational modification associated, among others, with diseases and the efficacy of biopharmaceuticals. Matrix-assisted laser desorption/ionization (MALDI) time-of-fight (TOF) mass spectrometry (MS) can be performed to study glycosylation in a high-throughput manner, but is hampered by the instability and ionization bias experienced by sialylated glycan species. Stabilization and neutralization of these sialic acids can be achieved by permethylation or by specific carboxyl group derivatization with the possibility of discrimination between α2,3- and α2,6-linked sialic acids. However, these methods typically require relatively pure glycan samples, show sensitivity to side reactions, and need harsh conditions or long reaction times. We established a rapid, robust and linkage-specific high-throughput method for sialic acid stabilization and MALDI-TOF-MS analysis, to allow direct modification of impure glycan-containing mixtures such as PNGase F-released human plasma N-glycome. Using a combination of carboxylic acid activators in ethanol achieved near-complete ethyl esterification of α2,6-linked sialic acids and lactonization of α2,3-linked variants, in short time using mild conditions. Glycans were recovered by hydrophilic interaction liquid chromatography solid phase extraction and analyzed by MALDI-TOF-MS in reflectron positive mode with 2,5-dihydroxybenzoic acid as the matrix substance. Analysis of the human plasma N-glycome allowed high-throughput detection and relative quantitation of more than 100 distinct N-glycan compositions with varying sialic acid linkages.



Protein glycosylation is a common post-translational modification having effects on characteristics such as solubility, folding and receptor binding activity.[1−3] N-glycans on mammalian proteins often exhibit terminal sialic acids such as *N*-acetylneuraminic acid, which is involved in binding to various human lectins, shows importance in cellular communication, and determines protein half-life.[4−6] Sialic acids are most frequently attached to a terminal galactose via α2,6 or α2,3 glycosidic linkage and show different functionality as a consequence. Examples include α2,3-linked *N*-acetylneuraminic acids being specifically required for formation of sialyl Lewis X structures, which have been found to be indicative of metastasis for several types of cancer,[7−10] whereas α2,6-linked sialic acids have been linked to promoting cell survival by galectin inhibition.[11−13] With glycosylation and specific features such as sialylation, galactosylation and fucosylation becoming increasingly interesting as a clinical biomarker reservoir, and a necessity for the characterization of biopharmaceuticals, the development of methods for high-throughput glycan analysis is required.

A technique well-suited for high-throughput glycomics is matrix-assisted laser desorption/ionization (MALDI) time-of-fight (TOF) mass spectrometry (MS), as it can rapidly provide profiles with information on glycan composition.[14] MALDI-TOF-MS analysis of sialylated glycans is, however, compromised by the labile nature of the sialic acid glycosidic bond, resulting in the facile loss of the residue by in-source and metastable decay. In addition, sialylated glycan species tend to show different salt adducts, resulting in multiple signals for single glycan compositions. Moreover, the carboxyl group present on a sialic acid preferentially facilitates negative ionization by MALDI, generating a bias in signal intensity when comparing acidic and neutral oligosaccharides.[15]

One way to improve MALDI-TOF-MS measurements of sialylated glycans is derivatization.[16,17] Commonly used is permethylation, which results in reproducible sialic acid stabilization as well as increased sensitivity of MS analysis but shows some variation in the number of methyl groups conveyed.[18−21] Another strategy leading to greatly enhanced stability involves the selective modification of the sialic acid carboxyl group.[15,16,22] Importantly, in contrast to permethylation, sialic acid-specific derivatization has been demonstrated in a manner dependent on the sialic acid linkage, with α2,6-linked sialic acids undergoing modifications such as esterification and amidation, whereas α2,3-linked sialic acids can instead form an

Received: January 24, 2014
Accepted: May 15, 2014
Published: May 15, 2014

   dx.doi.org/10.1021/ac500335t | *Anal. Chem.* 2014, 86, 5784−5793

intramolecular lactone, leading to water loss.[23−25] The resulting mass difference allows the direct distinction of sialic acid linkages by mass spectrometry. Methods described in literature to achieve this linkage-specific derivatization involve methyl esterification or (methyl)amidation, but these typically require highly purified glycan samples, harsh conditions or long reaction times, and often do not show complete derivatization and linkage specificity.[23−25] Although these procedures are informative for the analysis of sialylated oligosaccharides, suitability for high-throughput analysis of complex samples appears limited.

Here we present a fast, easy and mild protocol for linkage-specific sialic acid derivatization, using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) as activators in ethanolic solution. These chemicals are widely used in peptide synthesis, with EDC responsible for initial carboxylic acid activation and HOBt catalyzing the subsequent conversion to an ester or amide (Figure S-1, Supporting Information).[26−28] To arrive at the proposed conditions, several acid activators and alcohols were examined for reaction completeness and selectivity for sialic acid linkage. Repeatability of the final protocol was established within and across 3 days for PNGase F-released total plasma N-glycome and the suitability for high-throughput 96-well format analysis was demonstrated.

EXPERIMENTAL SECTION

Samples. The plasma pooled from 20 human donors (Visucon-F frozen normal control plasma, citrated and 0.02 M HEPES buffered) was obtained from Affinity Biologicals (Ancaster, Canada). Oligosaccharide standards 3′-sialyllactose (Neu5Acα2,3Galβ1,4Glc; Neu5Ac = *N*-acetylneuraminic acid; Gal = galactose; Glc = glucose) sodium salt and 6′-sialyllactose (Neu5Acα2,6Galβ1,4Glc) sodium salt (both with purities higher than 98%) were purchased from Carbosynth (Compton, U.K.). Both 3′- and 6′-sialyllactose were dissolved to a concentration of 100 mg/mL in water. Fibrinogen from human plasma was acquired from Sigma-Aldrich (Steinheim, Germany), and was incubated for 4.5 h at 37 °C in 1× PBS, resulting in a fibrinogen solution of 24.12 mg/mL.

Chemicals, Reagents and Enzymes. Milli-Q water (MQ) used in this study was generated from a Q-Gard 2 system (Millipore, Amsterdam, Netherlands), maintained at ≥18 MΩ. Methanol, ethanol, 2-propanol, 1-butanol, trifluoroacetic acid (TFA), glacial acetic acid, sodium dodecyl sulfate (SDS), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2H_2O$), potassium dihydrogen phosphate (KH_2PO_4) and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). *N*,*N*′-Dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt) hydrate, 2-aminobenzoic acid (2-AA), 2-picoline borane (2-PB), dimethyl sulfoxide (DMSO), 50% sodium hydroxide, 98% formic acid, 25% ammonium hydroxide in water and Nonidet P-40 (NP-40) were obtained from Sigma-Aldrich, whereas 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC) hydrochloride and ethyl-2-cyano-2-(hydroxyimino)acetate (trade name Oxyma Pure) originated from Fluorochem (Hadfield, U.K.). Additional components used for this study included recombinant peptide-N-glycosidase F (PNGase F) from Roche Diagnostics (Mannheim, Germany), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) from Santa Cruz Biotechnology (Santa Cruz, CA), 2,5-dihydroxybenzoic acid (2,5-DHB) from Bruker Daltonics (Bremen, Germany) and HPLC SupraGradient acetonitrile (ACN) from Biosolve (Valkenswaard, Netherlands).

N-Glycan Release. N-glycans were released from the human plasma and fibrinogen samples as described previously.[29] Ten microliters of sample was denatured by adding 20 μL of 2% SDS and incubating for 10 min at 60 °C. The release step was subsequently performed by adding 20 μL of release mixture containing 2% NP-40 and 0.5 mU PNGase F in 2.5× PBS (10× PBS containing 57 g/L $Na_2HPO_4 \times 2H_2O$, 5 g/L KH_2PO_4 and 85 g/L NaCl), and incubating overnight at 37 °C.

Cotton HILIC SPE Glycan Enrichment. Glycan enrichment was performed by cotton hydrophilic interaction liquid chromatography (HILIC) solid phase extraction (SPE) as described previously,[30] with a few modifications. Samples removed from −20 °C were allowed to return to room temperature before proceeding. Twenty microliter pipet tips (Rainin Instrument, Oakland, CA) were packed with 3 mm cotton thread (180 μg, Pipoos, Utrecht, Netherlands), which was then conditioned and equilibrated by pipetting three times 20 μL of MQ, followed by three times 20 μL of 85% ACN. The sample was loaded by pipetting 20 times into the reaction mixture, making sure not to include the sedimented precipitate, in order to prevent clogging of the tips. The tips were washed three times with 20 μL of 85% ACN 1% TFA, and three times with 20 μL of 85% ACN. Subsequent elution was performed in 10 μL of MQ. A 12-channel pipet was used for all steps.

96-Well Format Sepharose HILIC SPE. A 96-well format glycan enrichment was performed by Sepharose HILIC SPE, using previously reported conditions with some modifications.[31] Ten microliters of Cl-4B Sepharose beads (45−165 μm, GE Healthcare, Uppsala, Sweden) were added in 20% ethanol to a 96-well filter plate (0.7 mL/well, PE frit, Orochem, Naperville, IL), and solvent was removed by vacuum using a vacuum manifold (Millipore, Billerica, MA). Beads were washed three times with 180 μL of MQ and three times with 180 μL of 85% ACN, after which the samples were transferred to the plate. To facilitate binding, the plate was put on a multiwell plate shaker (1.5 mm orbit, VWR, Amsterdam, Netherlands) for 5 min at 1000 rpm, after which the remaining solvent was removed by vacuum. The plate was subsequently washed three times with 85% ACN 0.1% TFA and 85% ACN before applying 30 μL of MQ for elution. Again the plate was incubated for 5 min on a shaking platform at 1000 rpm, and elution performed by centrifuging 1 min at 200*g*.

MALDI-TOF-MS. For MALDI-TOF-MS analysis, 1 μL of glycan sample purified by HILIC SPE was spotted on a MTP AnchorChip 800/384 TF MALDI target (Bruker Daltonics, Bremen, Germany), mixed on plate with 1 μL 2,5-DHB (5 mg/mL) 1 mM NaOH in 50% ACN and left to dry by air. The matrix crystals were made uniform by addition of 0.2 μL ethanol, causing rapid recrystallization.

Analyses were performed RP-mode on an UltraFlextreme MALDI-TOF-MS equipped with a Smartbeam-II laser and Flexcontrol 3.4 software Build 119 (Bruker Daltonics). The instrument was calibrated using a peptide calibration standard (Bruker Daltonics, Bremen, Germany). A 25 kV acceleration voltage was applied after a 140 ns extraction delay. A mass window of m/z 100 to 1500 was used for α2,3- and α2,6-sialyllactose analysis, and a mass window of m/z 1000 to 5000 with suppression up to m/z 900 for fibrinogen and plasma N-glycan samples. For each spectrum, 20 000 laser shots were accumulated at a laser frequency of 2000 Hz, using a complete sample random walk with 200 shots per raster spot. High laser intensity was used for sample profiling to allow for ionization of

dx.doi.org/10.1021/ac500335t | *Anal. Chem.* 2014, 86, 5784−5793

larger glycan species, while the monoisotopic peak was still clearly defined for all detectable glycan masses.

Tandem mass spectrometry (MALDI-TOF/TOF-MS/MS) was performed on the most abundant peaks of human plasma N-glycome via laser-induced disassociation.

Analysis of MALDI-TOF-MS Data. Using flexAnalysis v3.3 build 65 (Bruker Daltonics), the acquired MALDI-TOF-MS spectra were internally recalibrated using a set of calibration masses (Table S-1, Supporting Information). More specifically, masses were calculated as $[M + Na]^+$, taking into account the different masses for the sialic acids, depending on their lactonization or alkylation. Glycan compositions used in the calibration of plasma N-glycome were H3N4F1, H4N4F1, H5N4F1, H5N4S1, H5N4F1S1, H5N4S2, H6N5S3, H6N5F1S3 and H7N6S4, (H = hexose, N = *N*-acetylhexosamine, F= fucose and S = *N*-acetylneuraminic acid with unspecified linkage, the number of residues given after the letter). For fibrinogen samples, only H5N4S1 and H5N4S2 were used for calibration, whereas for sialyllactose, the H2S1 values were calculated for alkylated and lactonized sialic acid variants. Masses were picked in the spectra using a centroid algorithm with a mass window of 0.5 Da, followed by quadratic calibration. Recalibrated spectra were exported as a text format and further analyzed using an in-house developed Python script (see the Supporting Information). In short, the analysis was performed as a targeted data extraction using a determined list of glycan compositions. For each composition, the isotopic distribution and its masses were calculated. For each isotopic peak within 95% of the cumulative isotopic peak pattern, the intensities were summed within a 1 Da mass window. Noise intensities were then summed for a 1 Da window located 1 Da lower than the isotope cluster and subtracted from each isotopic peak (resulting in the background-corrected signal values). Observed isotopic ratios were compared to the calculated ones to prevent errors due to overlap, after which the intensities for the individual isotopic peaks were summed per glycan composition. Percentages were calculated with standardization to the overall glycan signal intensity of each spectrum. Averages and standard deviations were calculated for repeated experiments. Figures were assigned with glycan structures using GlycoWorkbench 2.1 build 146.[32,33]

Activator Comparison. A selection of carboxylic acid activator reagents and their combinations was tested for suitability with sialic acid esterification of unpurified plasma N-glycome samples obtained from PNGase F treatment. DMT-MM, HOBt, Oxyma Pure, DCC and EDC were dissolved at 0.5 M in methanol, with and without 0.2% TFA. One microliter of released plasma N-glycome (containing approximating 14 μg of plasma proteins and the corresponding released N-glycans) was added to 20 μL of each reagent. In addition, 1 μL of released plasma N-glycome was added to 20 μL of methanol, containing the following reagent combinations: DCC with HOBt, DCC with Oxyma Pure, EDC with HOBt and EDC with Oxyma Pure, each component at a concentration of 0.25 M, and each condition with and without 0.2% TFA. All samples were incubated for 1 h at 60 °C. After the reaction, the samples were brought to an ACN concentration of 50% (v/v) by addition of 20 μL ACN, followed by 15 min incubation at −20 °C to stimulate additional precipitate formation. Cotton HILIC SPE was performed to achieve enrichment and purification of the derivatized glycans from the reaction mixture.

Comparison of Different Alcohols for Derivatization. 0.25 M EDC with 0.25 M HOBt was selected as the most promising reagent combination for sialic acid esterification, and additional alcohols besides methanol were tested for use as both solvent and alkyl donor. Solutions of 0.25 M EDC together with 0.25 M HOBt were prepared using four different alcohols: methanol, ethanol, 2-propanol and 1-butanol. One microliter of PNGase F-released plasma N-glycome was added to 20 μL of each of the four solutions, and the samples were incubated for 1 h at 60 °C. After this, 20 μL of ACN was added and the samples were incubated at −20 °C for 15 min before glycan enrichment by cotton HILIC and MALDI-TOF-MS analysis.

Linkage Specificity on α2,3- and α2,6-Sialyllactose. The linkage specificities of the 0.25 M EDC and 0.25 M HOBt solutions in both methanol and ethanol were tested using oligosaccharide standards with a known sialic acid linkage position. One microliter of either 100 mg/mL (100 μg) α2,3- or α2,6-sialyllactose was added to 20 μL of methanol or ethanol containing 0.25 M EDC and 0.25 M HOBt. Samples were incubated for 1 h at 60, 50, 37, 21 or 4 °C. After the addition of 20 μL of ACN, all samples were incubated for 15 min at −20 °C before proceeding with glycan enrichment and analysis.

Repeatability Testing. Repeatability of the established ethyl esterification conditions was ascertained by multiple analyses of the same sample across several days, performing all steps in a 96-well polypropylene PCR plate (Greiner Bio-One), sealed with adhesive tape (Nutacon, Leimuiden, Netherlands) during incubation steps. For each day, 24 independent samples were taken from the same pooled plasma and deglycosylated with PNGase F as described before. One microliter of each released sample was ethyl esterified by adding it to a new PCR plate containing 20 μL of 0.25 M HOBt 0.25 M EDC in ethanol and incubating the solution for 1 h at 37 °C. Twenty microliters of ACN was then added and the plate incubated at −20 °C for 30 min. Samples were purified by cotton HILIC SPE and measured by MALDI-TOF-MS. The entire procedure was repeated twice on consecutive days with freshly prepared reagents to establish day-to-day variability.

Fibrinogen Glycan Labeling. Aliquots of PNGase F-released N-glycans from fibrinogen were modified by either ethyl esterification or fluorescent labeling of the reducing end with 2-AA to allow for a comparison between MALDI-TOF mass spectrometric data and profiles obtained by HILIC ultra high-performance liquid chromatography (UHPLC) with fluorescent detection. For ethyl esterification, the optimal conditions were established in previous experiments. One microliter of fibrinogen release mix (corresponding to 24.12 μg protein) was added to 20 μL of 0.25 M EDC and 0.25 M HOBt in ethanol, and incubated for 1 h at 37 °C. Subsequently, 20 μL of ACN was added and the mixture was stored at −20 °C for 15 min before glycan enrichment and MALDI-TOF-MS analysis.

For HILIC-UHPLC, the released N-glycans were labeled with 2-AA as described previously.[34] In short, 20 μL of PNGase F-released fibrinogen glycans was mixed with 10 μL of 2-AA (48 mg/mL) in DMSO with 15% glacial acetic acid and 10 μL of 2-PB (107 mg/mL) in DMSO. The mixture was incubated for 2 h at 65 °C and diluted to 75% ACN before HILIC-UHPLC analysis.

HILIC-UHPLC Measurement. For the separation and analysis of 2-AA labeled N-glycans, HILIC-UHPLC with fluorescent detection was performed on a Dionex Ultimate 3000 (Thermo Fisher Scientific, Breda, Netherlands) equipped with a 1.7 μm × 2.1 × 100 mm Acquity UPLC BEH Glycan column (Waters, Etten-Leur, Netherlands). Separation was performed at 60 °C with a flow rate of 0.6 mL/min. Two solutions were used for gradient generation, ACN as solution A

dx.doi.org/10.1021/ac500335t | *Anal. Chem.* 2014, 86, 5784−5793

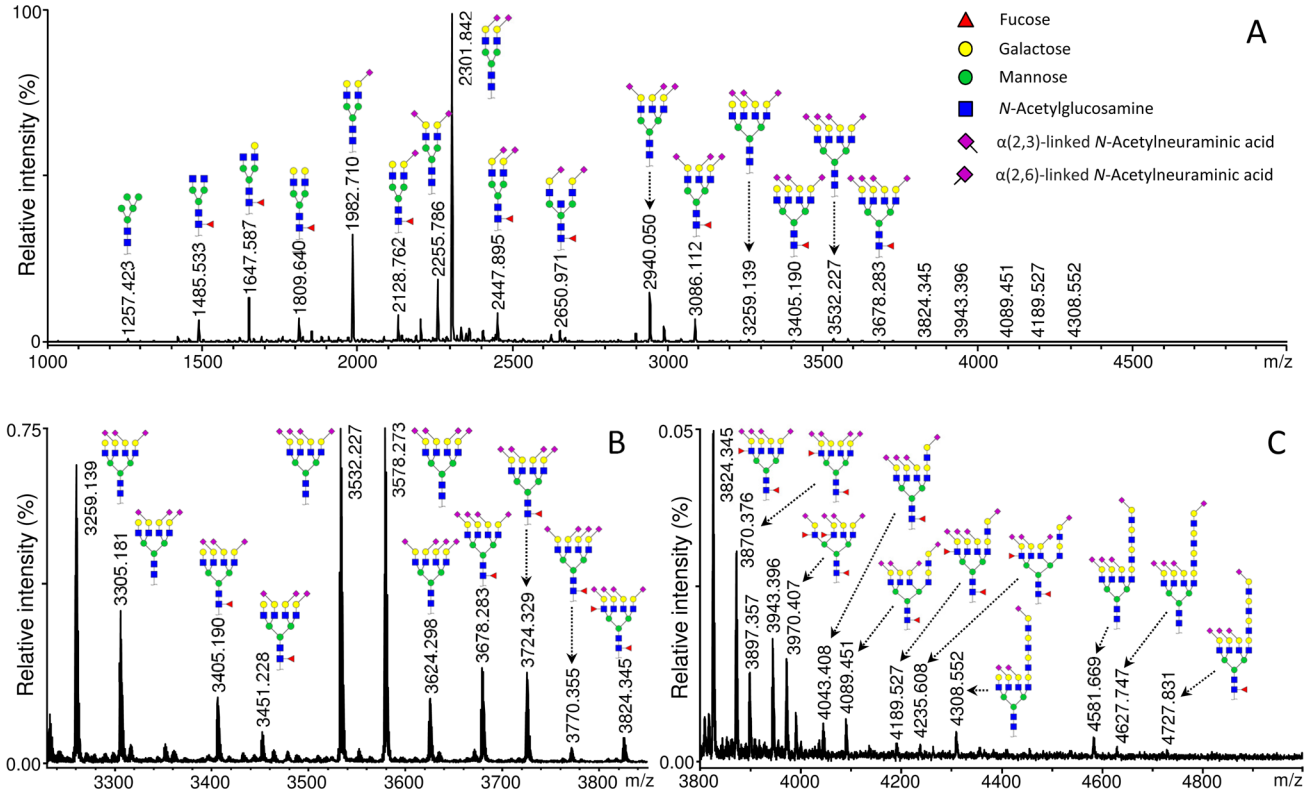


Figure 1. RP MALDI-TOF-MS spectrum after applying the 1 h at 37 °C EDC+HOBt ethyl esterification protocol for plasma N-glycome analysis. (A) Overview spectrum. (B) Intermediate m/z range spectrum (m/z 3200 to 3800). (C) High m/z range spectrum (m/z 3800 to 5000) showing glycan masses of up to 4727.640 Da $[M + Na]^+$. Proposed glycan structures are based on literature,[36,41−43] as well as MS/MS data (Table S-2, Supporting Information). Structures are depicted following the CFG notation. Linkage positions of sialic acid residues are indicated by differing angles.

and 100 mM ammonium formate pH 4.4 (prepared as formic acid buffered to pH 4.4 by NH_4OH) as solution B. The column was equilibrated by 85% solution A for 0.5 min. After this, the samples were loaded in 75% A, and the excess fluorescent reagent was eluted from the column by washing with 85% A for 10 min. The separation gradient started at 75% A and decreased linearly to 57% A in 45 min. The column was then flushed again with 40% A for 10 min followed by 10 min of 85% A for re-equilibration. For fluorescent detection, 330 nm was used for excitation and the emission recorded at 420 nm. The resulting chromatograms were analyzed using Chromeleon version 7.1.2.1713 (Thermo Fisher Scientific).

RESULTS

A robust, high-throughput MALDI-TOF-MS method was developed for profiling human plasma N-glycans with linkage-specific derivatization of sialic acid residues. Reaction conditions were optimized to achieve ethylation of α2,6-linked sialic acid residues with parallel lactone formation of α2,3-linked sialic acids. Commercially available pooled plasma (10 μL) was subjected to PNGase F treatment to obtain a complex sample with free N-glycans. The resulting plasma N-glycome contains a large set of N-glycans of different compositions, including neutral as well as highly sialylated species with varying linkages,[35,36] making it a demanding sample to study the overall effects of sialic acid modification methods. Ethylation using carboxylic acid activators EDC and HOBt was chosen due to its high catalytic efficacy and specificity. Glycan profiles were obtained, allowing the differentiation of 77 glycan compositions, which resulted in 108 glycan species due to variation in sialic acid linkage (Figure 1). Method development and validation are described in the following sections.

Comparison of Different Activators. A number of coupling reagents as well as combinations thereof were compared for linkage-specific esterification of sialic acid residues in impure mixtures containing native N-glycans. All reactions were performed directly on the unpurified PNGase F released N-glycan mixture, using methanol as both methyl donor and solvent. Samples were allowed to react for 1 h at 60 °C in methanol containing coupling/activation reagents DMT-MM, DCC, EDC, HOBt or Oxyma Pure, each tested with and without 0.2% TFA. Additionally, reagent combinations DCC + HOBt, DCC + Oxyma Pure, EDC + HOBt and EDC + Oxyma Pure were tested, each with and without 0.2% TFA. During the 1 h incubation step, a white precipitate formed at the bottom of the samples, causing separation between the methanol soluble components within the mixture (including glycans and the coupling reagents), and the methanol insoluble fraction containing proteins.

Samples were purified by cotton HILIC SPE. To this purpose, various volumes of ACN were added, and concentrations between 25 and 75% were found to result in excellent enrichment without noticeable bias for low- or high-mass species. The intermediate value of 50% ACN was chosen for purification. One microliter of the purified samples was spotted on an AnchorChip MALDI plate and cocrystallized with 1 μL of 2,5-DHB (5 mg/mL) as the matrix. To suppress potassium adduct formation, 1 mM NaOH was added to the matrix solution, yielding almost exclusively $[M + Na]^+$ species in reflectron positive mode. Recrystallization was performed with 0.2 μL ethanol to decrease

dx.doi.org/10.1021/ac500335t | Anal. Chem. 2014, 86, 5784−5793

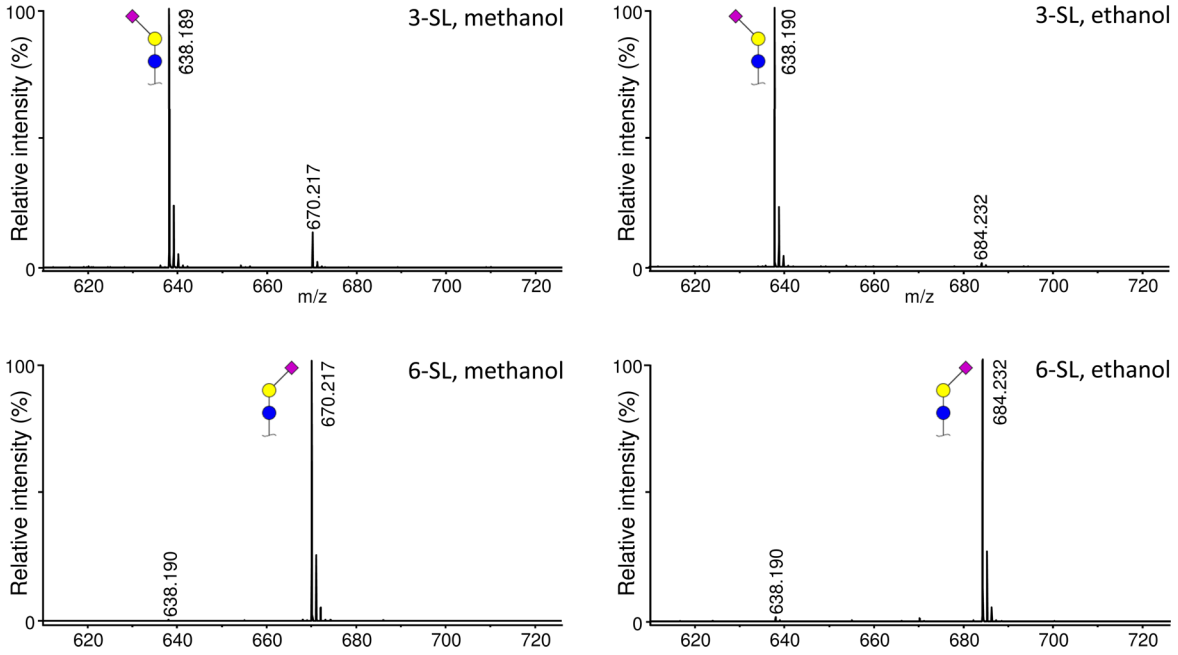


Figure 2. RP MALDI-TOF-MS spectra of 3′- and 6′-sialyllactose when alkyl esterified for 1 h at 37 °C with EDC + HOBt in methanol or ethanol. The lactonized reaction product is visible at 638.190 Da $[M + Na]^+$, with the esterified masses being 670.217 and 684.232 Da for methanol and ethanol, respectively. In no case was the mass of 678.183 Da $[M - H + 2Na]^+$ visible, which would be indicative for incomplete reaction. Reaction in ethanol shows the highest specificity for sialic acid linkage with undesired reaction products only appearing in minimal amounts.

shot-to-shot variation and further decrease the variation in salt adduct formation.

Efficacy of sialic acid derivatization was determined for the various reaction conditions by evaluating the signals of the sialylated N-glycans (Figure S-2, Supporting Information). The mass shift induced by the desired methyl esterification is +14.016 Da per sialic acid, resulting in a mass of 2273.823 Da $[M + Na]^+$ for the fully esterified major plasma N-glycan H5N4S2. Glycans with unmodified sialic acids were observed in RP mode with full exchange of proton for sodium on the carboxylic acid groups (+21.982 Da per sialic acid), resulting in signals at m/z 2281.763 and 2289.740, indicative of incomplete reactions. In addition, lactonization of one of the sialic acids could be observed, yielding an 18.011 Da mass loss, resulting in a signal at m/z 2241.767 $[M + Na]^+$.

Single reagents DCC, EDC, HOBt and Oxyma Pure proved unable to induce modification of sialic acids, regardless of whether acid was used during the reaction or not. DMT-MM did show methyl esterification of sialic acid residues, but also generated a prominent peak at m/z 2240.811, as well as a signal at m/z 2258.817 (amidation of carboxylic acid groups, resulting in a −0.984 Da mass difference per acid). These side products were largely prevented by adding 0.2% TFA to the reaction, but strong acidic conditions may also result in partial desialylation and loss of other labile substituents. Reagent combinations DCC + HOBt, DCC + Oxyma Pure and EDC + Oxyma pure resulted in prominent products with methyl esterification of the sialic acid residues, but the presence of sodium adducts also indicated the conversion to be incomplete. This was largely, but not completely, corrected by using acidified reaction mixtures. EDC + HOBt appeared to be the most promising reagent combination, showing complete conversion regardless of the use of acid, and this conditions was therefore selected for further experimentation.

Testing of Different Alcohols. EDC + HOBt was selected as the most promising reagent mixture for sialic acid esterification, and various alcohols were tested as combined protein precipitation agent and alkyl donor. After a solution of 0.25 M EDC and 0.25 M HOBt in 20 μL of methanol, ethanol, 2-propanol or 1-butanol was added to 1 μL of plasma N-glycome solution, the reaction was performed for 1 h at 60 °C. EDC and HOBt dissolved only partially in 2-propanol and 1-butanol at room temperature; therefore, a well-homogenized suspension was used instead. Purification of the reaction products and analysis by RP MALDI-TOF-MS were performed as described above.

Analysis of sialylated glycan compositions showed all alcohols to be an alkyl group donor for sialic acid esterification (Figure S-3, Supporting Information). Trisialylated glycan compositions H6N5S3 and H6N5F1S3 were studied as examples. Expected masses after derivatization with methanol, ethanol, 2-propanol and 1-butanol were 2944.047, 2986.094, 3028.141 and 3070.188 Da for composition H6N5S3 and 3090.105, 3132.152, 3174.199 and 3216.246 Da for H6N5F1S3, all observable in the respective spectra. In addition, lactonization products were present in all spectra, with mass differences of 32.026, 46.042, 60.058 and 74.073 Da as compared to the alkyl esterification products, but relative ratios of the lactonized and alkyl esterified species differed per alcohol. 2-Propanol showed a large variety of reaction products in addition to the expected species, and both 2-propanol and 1-butanol yielded relatively low signals for the sialylated glycans. Methanol and ethanol were both selected as the most promising solvents and alcohol donors for linkage-specific sialic acid modification and were therefore used for further method development.

Achieving Linkage Specificity. The linkage specificity of EDC + HOBt in combination with methanol and ethanol was studied using oligosaccharide standards with known sialic acid linkage, namely 3′-sialyllactose (Neu5Acα2,3Galβ1,4Glc) and

dx.doi.org/10.1021/ac500335t | Anal. Chem. 2014, 86, 5784−5793

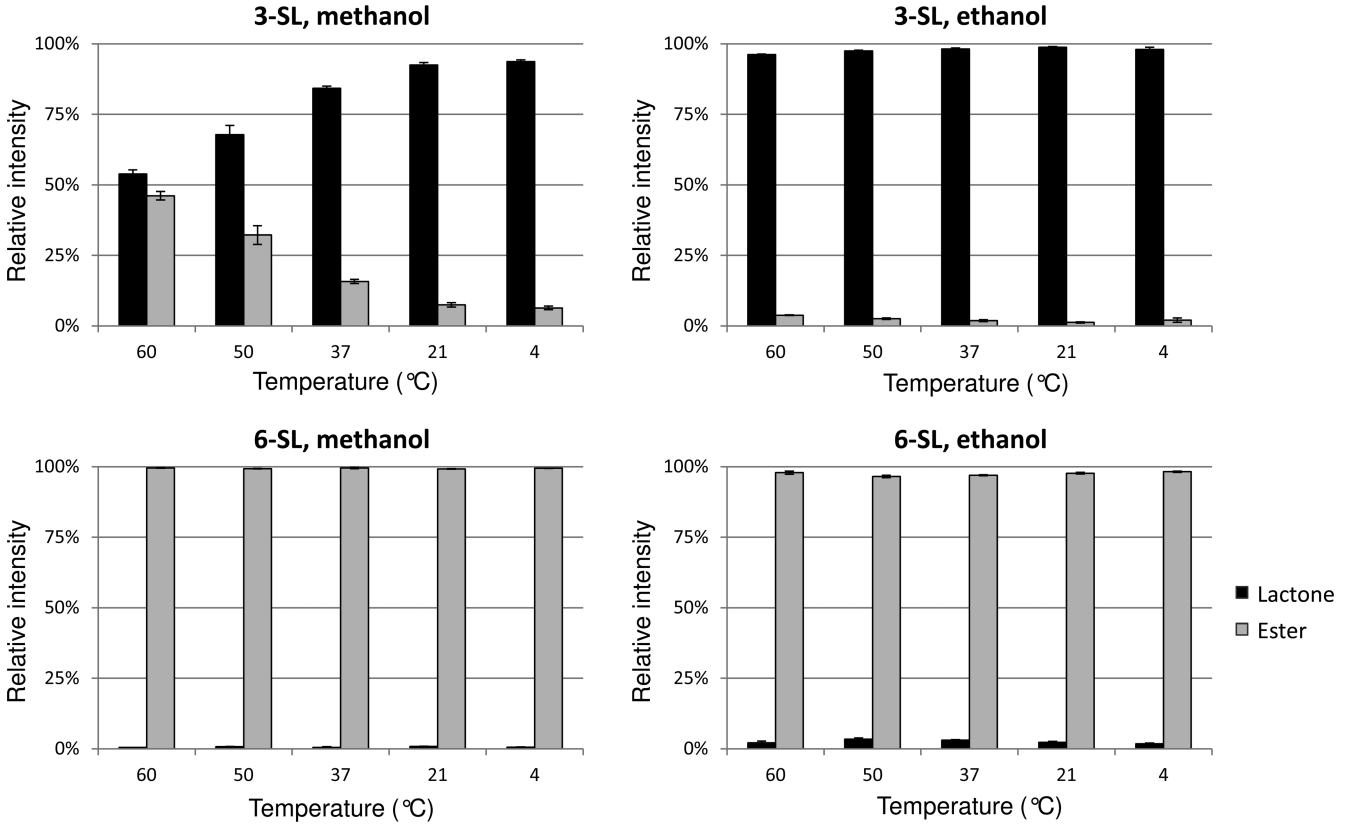


Figure 3. Triplicate analysis of 3′- and 6′-sialyllactose after EDC + HOBt alkylation in either methanol or ethanol, at varying temperatures. Shown in the graphs are the average relative intensities of the lactonized and esterified reaction products, with the error bars indicating standard deviation. α2,6-Linked sialic acids show to preferably form an ester with both methanol and ethanol at all temperatures. Reacting α2,3-linked sialic acids in methanol, however, shows a temperature dependent effect, at best 93.6% (SD ± 0.9%) of the desired lactonized product being formed at 4 °C. Using ethanol as the alkyl donor, on the other hand, shows on average 97.7% (SD ± 0.9%) lactonization across all temperatures and is therefore most suitable for separating α2,3- and α2,6-linked sialic acids.

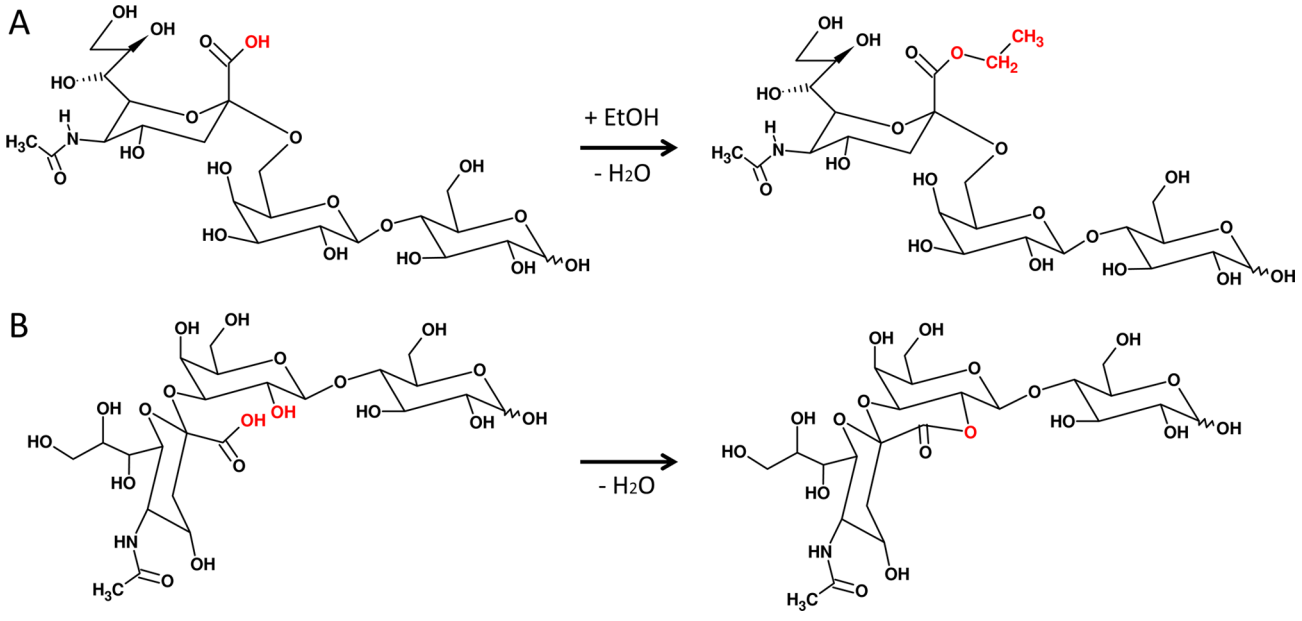


Figure 4. Structures of (A) 6′-sialyllactose and (B) 3′-sialyllactose before and after ethyl esterification. Whereas α2,6-linked sialic acids show ethyl esterification of the carboxylic acid, α2,3-linked sialic acids instead form a lactone with the neighboring galactose.

6′-sialyllactose (Neu5Acα2,6Galβ1,4Glc), both with purities higher than 98%. To study the temperature effect on linkage-specificity, the samples were reacted not only for 1 h at 60 °C but also at 50, 37, 21 and 4 °C. Purification of reaction products and RP MALDI-TOF-MS analysis were performed as before (Figure 2).

dx.doi.org/10.1021/ac500335t | Anal. Chem. 2014, 86, 5784−5793

For each experiment, relative quantitation of the lactonized reaction products (638.190 Da in all conditions) and the methyl- and ethyl esterified products (670.217 and 684.232 Da, respectively) was performed (Figure 3). The unmodified glycan was not found (expected signal for $[M - H + 2Na]^+$ at 678.183 Da), indicating that all sialic acids were either esterified or lactonized. α2,6-Linked sialic acids were shown to be highly susceptible to alkylation, as both methanol and ethanol led to a near complete esterification. However, lactonization of α2,3-linked sialic acids in methanol displayed a temperature dependent conversion, with a lower amount of side reaction (methanol esterification) formed at lower temperatures. Using methanol, correct lactonization ranged from only 53.9% (standard deviation (SD) ± 1.5%) at 60 °C to 93.6% (SD ± 0.6%) at 4 °C. The use of ethanol, however, resulted in a much higher preference for α2,3-linked sialic acid lactonization, showing, on average, only 2.3% (SD ± 0.9%) of side reaction (ethanol esterification) across all temperatures. Because of the nearly complete selectivity between 3′- and 6′-sialyllactose reaction products (Figure 4), ethanol was selected as the solvent and donor for linkage-specific sialic acid modification by EDC + HOBt. Reaction conditions were set at 1 h at 37 °C for further experiments, to allow for a controlled temperature condition that is readily available in most laboratories.

Sialic Acid Stability. The ratios of H5N4S1 and H5N4S2 glycans released from fibrinogen were determined by MALDI-TOF-MS after ethyl esterification and compared with ratios determined after 2-AA labeling by HILIC-UHPLC and fluorescence detection. Triplicate analysis and relative quantification of the monosialylated and disialylated glycans showed signals averaging on 65.3% and 34.7% (SD ± 1.5%) for MALDI-TOF-MS and 66.4% and 33.6% (SD ± 0.2%) for the UHPLC measurements (Figure 5). The standard deviation of the MALDI method was noticeably higher than that for the UHPLC method, with a coefficient of variation (CV) of 4.3% for the smaller peak and 2.3% for the larger peak. Importantly, the proportions of the major monosialylated and disialylated biantennary glycans proved to be highly similar between mass spectrometry and UHPLC, and correspond with the literature.[37−40]

Repeatability. Multiple repeat analyses were performed to demonstrate the repeatability of EDC + HOBt ethyl esterification (1 h at 37 °C) for human plasma N-glycome profiling. The control plasma was divided into 24 separate samples, glycans were released with PNGase F, ethyl esterified, purified by cotton HILIC SPE and analyzed by RP MALDI-TOF-MS. Glycan signals were integrated, normalized to the sum of intensities, and the relative signals and standard deviations were calculated. This protocol was performed for two additional days to account for the day-to-day variation.

Repeatability analysis for the 20 most abundant glycans (responsible for 94% of the cumulative glycan distribution of a spectrum) across 24 independent samples originating from the same plasma pool revealed high repeatability within one batch, as well as between three batches prepared and measured on different days. Average relative intensity values for the highest peak (H5N4E2, the two esterified sialic acids (E) indicating α2,6-linkage) were around 54.7% (SD ± 2.3%) across all measurements, with the CV averaging to 3.8% (Figure 6). Analysis of all assigned glycan masses shows low variability even for values below 0.1% of the cumulative distribution, including those in very high mass ranges (Figure S-4, Supporting Information).

96-Well Plate Protocol. To validate the ethyl esterification protocol for applicability in high-throughput format, all steps were performed on a 96-well plate, employing Sepharose HILIC SPE rather than cotton purification. MALDI-TOF-MS spectra obtained after Sepharose were similar to those obtained from cotton and high quality spectra could be obtained across the plate (Figure S-5, Supporting Information). Relative quantification revealed only slight variations between the two HILIC modes, noticeable in the H5N4E1, H5N4E1L1 and H5N4E2 compositions.

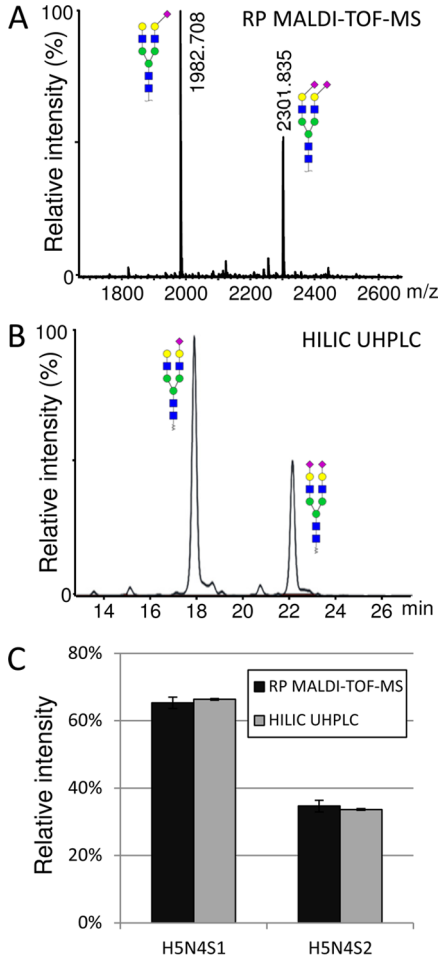


Figure 5. Released fibrinogen N-glycans as studied by (A) RP MALDI-TOF-MS after 1 h 37 °C EDC + HOBt ethyl esterification, and (B) 2-AA labeling and HILIC UHPLC with fluorescent detection. (C) Triplicate analysis and relative quantification of the mono- and disialylated structures shows highly comparable relative signal intensities between MALDI-TOF-MS and UHPLC analysis. Abbreviations used are hexose (H), *N*-acetylhexosamine (N), fucose (F), and *N*-acetylneuraminic acid with unspecified linkage (S). The accompanying number indicates the number of residues. The error bars show standard deviations.

Structural Assignment. Plasma profiles studied using the ethyl esterification protocol showed glycans $[M + Na]^+$ ranging in mass from 1257.423 Da (H5N2) to 4727.640 Da (H10N9F1 with four lactonized (L) sialic acids indicating α2,3-linkage). In total, 217 isotope clusters could be detected in the spectra, 108 of which could be attributed to specific glycan compositions within a low mass deviation (average mass deviation 0.015 Da), accounting for approximately 90% of the total spectrum intensity (Table S-2, Supporting Information). The most commonly found signals that could not be assigned to a native glycan structure showed masses 101.051 and 221.090 Da lower than the

dx.doi.org/10.1021/ac500335t | *Anal. Chem.* 2014, 86, 5784−5793

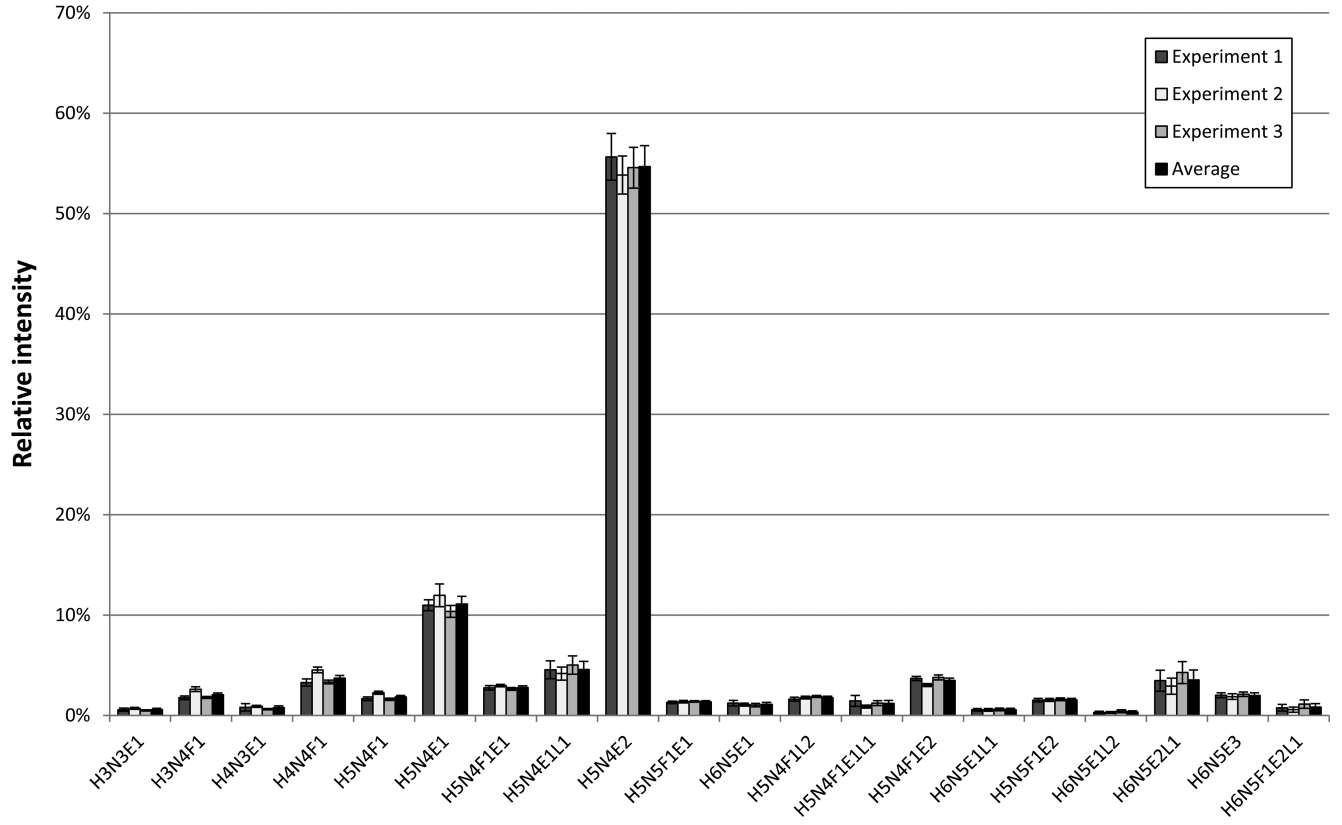


Figure 6. Repeatability of plasma N-glycan profiling by MALDI-TOF-MS after 1 h at 37 °C EDC + HOBt ethyl esterification. Twenty-four samples originating from a stock of control plasma were independently released by PNGase F, ethyl esterified, purified by HILIC SPE, crystallized with matrix, recrystallized with ethanol and analyzed by RP MALDI-TOF-MS. The experiment was performed three times on separate days, indicated as experiments 1−3. The graph shows the average relative intensities observed for the 20 major glycan species (normalized to the overall sum of intensities), with error bars for standard deviation. Abbreviations used are hexose (H), *N*-acetylhexosamine (N), fucose (F) and *N*-acetylneuraminic acid with either unspecified linkage (S), α2,3-linkage as indicated by lactonization (L) or α2,6-linkage as indicated by esterification (E).

associated major glycan peaks, respectively indicating an ^{0,2}A cross-ring fragmentation or complete loss of the reducing end *N*-acetylglucosamine. The relative abundances of these fragments increase with increasing laser power (respectively, around 4.3% and 0.3% of the major peak for low laser power settings and 9.6% and 0.7% for high laser power settings) but do not seem to discriminate between specific glycan types. Another side-reaction, which could arise at +357.184 Da from major peaks, may be attributable to a yet unidentified reducing end modification. Altogether, these unwanted products are minor and do not overlap or change the relative intensities of glycans in the spectrum, allowing analysis of glycan profiles without interference.

Glycans were structurally assigned on the basis of literature knowledge on the human plasma N-glycome and established biosynthetic pathways,[36,41−43] together with analytical data acquired in this study. MALDI-TOF/TOF-MS/MS was performed on the 38 highest intensity glycan peaks in the plasma N-glycome spectrum in order to confirm compositions and derive key structural features (Table S-2, Supporting Information). The observed fragmentation spectra proved informative with regard to antenna composition and fucosylation (Figure 7). Particulary high intensity fragments included the loss of the reducing end *N*-acetylglucosamine (221.090 Da), which was observed in all spectra, except for the spectra of core-fucosylated precursors which instead showed the loss of the reducing end *N*-acetylglucosamine with fucose (367.148 Da). Furthermore, sialylated glycans showed high intensity signals at losses of 638.217 Da (antenna carrying an α2,3-linked sialic acid), 684.259 Da (antenna carrying an α2,6-linked sialic acid) or 784.275 Da (antenna carrying an α2,3-linked sialic acid with additional fucose). The latter was only assigned as a fucosylated sialylated antenna if an accompanying loss of 221.090 Da would indicate lack of core fucosylation, and a 638.217 Da fragment could not be seen. No evidence was obtained for fucosylation of α2,6-sialylated antennae.

DISCUSSION

In this study, we present a high-throughput glycan derivatization method for MALDI-TOF-MS analysis, which allows the stabilization of sialylated species in a linkage-specific manner. Although the differential reactivity of α2,3- and α2,6-linked sialic acids has previously been reported, as well as derivatization methods relying on this feature,[23,24] the ethanol esterification described in this article shows marked advantages over current literature. First, we set up a one-pot approach resulting in a rather simple protocol with minimized sample handling steps. This allowed us to establish a robust high-throughput method in 96-well plate format. Second, we succeeded in increasing the specificity of the reaction by choosing efficient activators and mild reaction conditions: therefore, the presence of detergents, salts and amines, at the levels found in plasma glycan release samples, did not noticeably impede the specific derivatization of sialic acids by ethyl ester formation and lactonization. Third, the chemicals needed in the procedure are inexpensive and of only

dx.doi.org/10.1021/ac500335t | *Anal. Chem.* 2014, 86, 5784−5793

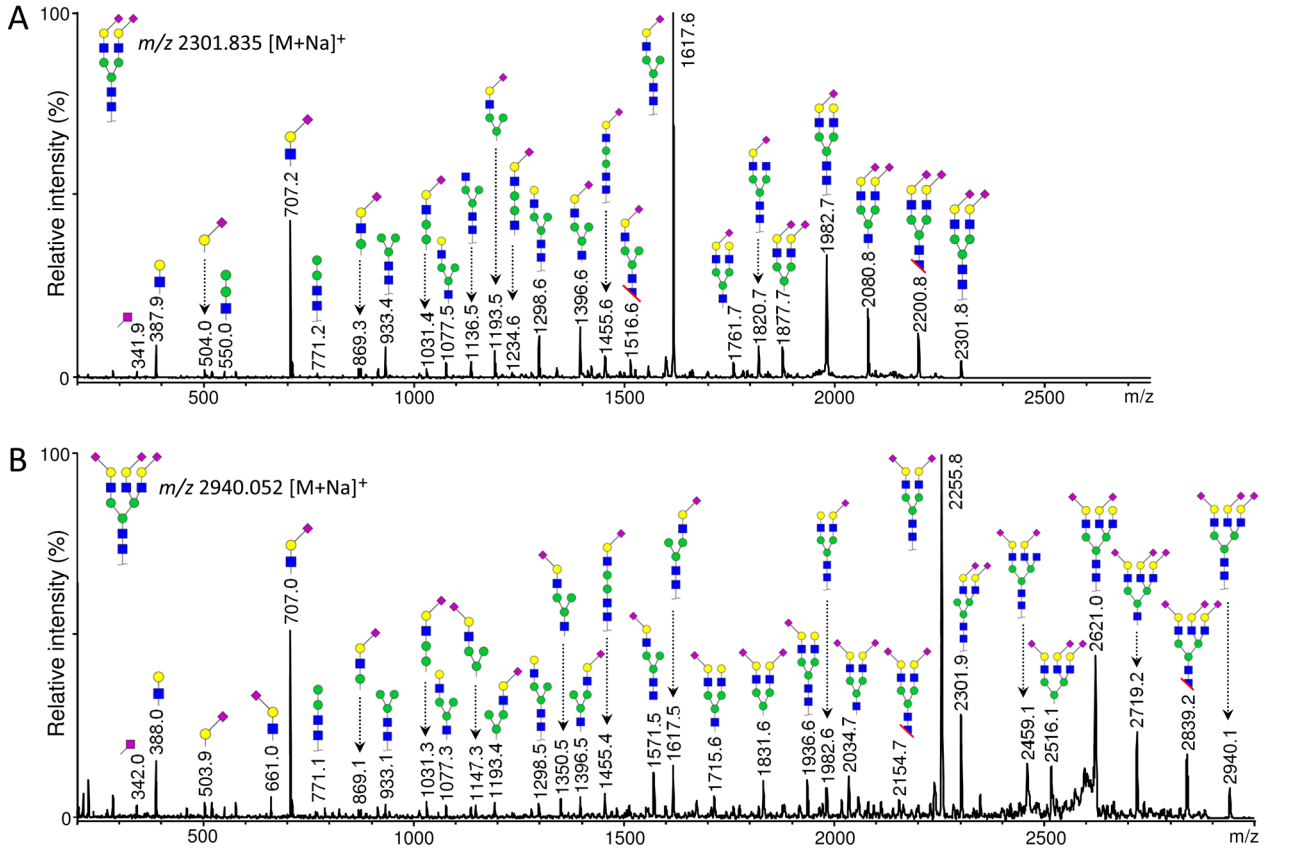


Figure 7. MALDI-TOF/TOF-MS/MS spectra of two major glycans of the ethyl esterified plasma N-glycome. (A) Biantennary species carrying two α2,6-linked sialic acids. (B) Triantennary species carrying two α2,6-linked sialic acids and one α2,3-linked sialic acid. Prominent losses include the α2,6-sialylated arms (684.3 Da), which can be observed in both spectra (resulting in signals at m/z 1617.6 and 2255.8, respectively), as well as the unique loss of an α2,3-sialylated arm (638.2 Da) in the triantennary spectrum (m/z 2301.9). Additional common losses include the loss of the reducing end *N*-acetylglucosamine (221.1 Da, visible at m/z 2080.8 and 2719.2, respectively) and an ^{0,2}A crossring fragment thereof (101.1 Da, visible at m/z 2200.8 and 2839.2, respectively) (indicated by a red line).

low toxicity, increasing the attractiveness of the method for routine, high-throughput applications.

We succeeded in performing ethyl esterification directly on impure samples like PNGase F-released plasma N-glycome, which may be attributed to several factors. Using ethanol as a solvent induces precipitation of proteins and peptides, reducing their interference with the reaction, while the glycans remain soluble and available for modification.[44] Not surprisingly, when the ethylation reaction was performed with shaking instead of at a stable position, we witnessed a significant decrease in reaction completeness (not shown). Another reason could be the excess of ethanol in the reactive environment. Esterification of α2,6-linked sialic acids with ethanol hereby becomes a high probability event, while steric hindrance still allows full lactonization of α2,3-linked sialic acids. Glycan samples modified by the ethyl esterification show markedly improved spectra over native ones in reflectron positive MALDI-TOF-MS. The resulting spectrum of total human plasma N-glycans is largely in line with patterns determined by more quantitative chromatographic methods.[35,45]

The mass changes induced by the sialic acid derivatization method allow the discrimination of α2,3- and α2,6-linked sialic acids directly from the overview spectrum and, contrary to underivatized samples, separate the isotopic peak patterns of sialylated and difucosylated glycan species (respectively, 291.095 and 292.116 Da mass increment without derivatization). However, while the current protocol is optimized for the modification of α2,3- and α2,6-linked *N*-acetylneuraminic acids, the efficacy of related derivatization schemes for other acidic residues present on glycans remains unknown. Notable examples include glucuronic acid, which has recently been found on human plasma N-glycans,[45] as well as monosaccharide modifications like phosphorylation and sulfation. In addition, whereas Neu5Acα2,3Gal and Neu5Acα2,6Gal can be discriminated by the proposed method, the reactivities of *N*-glycolylneuraminic acids, α2,8-linked *N*-acetylneuraminic acids, *O*-acetylated sialic acids and sialic acids linked to N-acetylhexosamines remain to be investigated.

To conclude, we have introduced a facile method for linkage-specific derivatization of sialic acid residues, using the combination of EDC and HOBt in ethanol, to allow for enhanced glycan detection by MALDI-TOF-MS. The method is characterized by high linkage specificity, overall mild and short reaction conditions, resistance to impurities, low cost and toxicity and repeatability within and between batches of samples. Altogether, we expect the proposed ethyl esterification to be an excellent method for the high-throughput analysis of sialylated glycans derived from biological sources and biopharmaceuticals.

■ ASSOCIATED CONTENT

Supporting Information

Figures containing the reaction mechanism of ethyl esterification, RP MALDI-TOF-MS analysis of PNGase F-released plasma N-glycome after methyl esterification, RP MALDI-

dx.doi.org/10.1021/ac500335t | *Anal. Chem.* 2014, 86, 5784−5793

TOF-MS of the plasma N-glycome after esterification with methanol, ethanol, 2-propanol and 1-butanol as solvents and alkyl donors using EDC + HOBt activators, repeatability of plasma N-glycan profiling by MALDI-TOF-MS after 1 h 37 °C EDC + HOBt ethyl esterification, RP MALDI-TOF-MS example spectrum of Sepharose purified plasma N-glycans after ethyl esterification, comparison of relative intensities obtained from cotton HILIC and Sepharose HILIC plate purification procedures, and an Excel workbook containing glycan signal assignments. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Manfred Wuhrer. Address: Center for Proteomics and Metabolomics, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands. E-mail: m.wuhrer@lumc.nl. Tel: +31-71-52-66396.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the European Union (Seventh Framework Programme HighGlycan project, grant number 278535). The authors thank Emanuela Lonardi for critically reading the paper, and David Falck for his assistance with the displayed reaction mechanism.

REFERENCES

(1) Wormald, M. R.; Dwek, R. A. *Structure* **1999**, *7*, R155−60.
(2) Crocker, P. R.; Paulson, J. C.; Varki, A. *Nat. Rev. Immunol.* **2007**, *7*, 255−66.
(3) Muramatsu, T. *J. Biochem* **2000**, *127*, 171−6.
(4) Dall'Olio, F.; Chiricolo, M. *Glycoconjugate J.* **2001**, *18*, 841−50.
(5) Schauer, R. *Curr. Opin. Struct. Biol.* **2009**, *19*, 507−14.
(6) Sperandio, M.; Gleissner, C. A.; Ley, K. *Immunol. Rev.* **2009**, *230*, 97−113.
(7) Isozaki, H.; Ohyama, T.; Mabuchi, H. *Int. J. Oncol.* **1998**, *13*, 935−42.
(8) Nakayama, T.; Watanabe, M.; Katsumata, T.; Teramoto, T.; Kitajima, M. *Cancer* **1995**, *75*, 2051−6.
(9) Jeschke, U.; Mylonas, I.; Shabani, N.; Kunert-Keil, C.; Schindlbeck, C.; Gerber, B.; Friese, K. *Anticancer Res.* **2005**, *25*, 1615−22.
(10) Jorgensen, T.; Berner, A.; Kaalhus, O.; Tveter, K. J.; Danielsen, H. E.; Bryne, M. *Cancer Res.* **1995**, *55*, 1817−9.
(11) Schultz, M. J.; Swindall, A. F.; Bellis, S. L. *Cancer Metastasis Rev.* **2012**, *31*, 501−18.
(12) Zhuo, Y.; Bellis, S. L. *J. Biol. Chem.* **2011**, *286*, 5935−41.
(13) Hsu, D. K.; Yang, R. Y.; Liu, F. T. *Methods Enzymol.* **2006**, *417*, 256−73.
(14) Harvey, D. J. *Mass Spectrom. Rev.* **1999**, *18*, 349−450.
(15) Powell, A. K.; Harvey, D. J. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1027−32.
(16) Harvey, D. J. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2011**, *879*, 1196−225.
(17) Ruhaak, L. R.; Zauner, G.; Huhn, C.; Bruggink, C.; Deelder, A. M.; Wuhrer, M. *Anal Bioanal. Chem.* **2010**, *397*, 3457−81.
(18) Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209−217.
(19) Morelle, W.; Michalski, J. C. *Nat. Protoc.* **2007**, *2*, 1585−602.
(20) Kang, P.; Mechref, Y.; Klouckova, I.; Novotny, M. V. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 3421−8.
(21) Kang, P.; Mechref, Y.; Novotny, M. V. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 721−34.
(22) Miura, Y.; Shinohara, Y.; Furukawa, J.; Nagahori, N.; Nishimura, S. *Chemistry* **2007**, *13*, 4797−804.
(23) Wheeler, S. F.; Domann, P.; Harvey, D. J. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 303−12.
(24) Alley, W. R., Jr.; Novotny, M. V. *J. Proteome Res.* **2010**, *9*, 3062−72.
(25) Liu, X.; Qiu, H.; Lee, R. K.; Chen, W.; Li, J. *Anal. Chem.* **2010**, *82*, 8300−6.
(26) Neises, B.; Steglich, W. *Angew. Chem.* **1978**, *17*, 522−524.
(27) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788−798.
(28) Chan, L. C.; Cox, B. G. *J. Org. Chem.* **2007**, *72*, 8863−9.
(29) Ruhaak, L. R.; Huhn, C.; Waterreus, W. J.; de Boer, A. R.; Neususs, C.; Hokke, C. H.; Deelder, A. M.; Wuhrer, M. *Anal. Chem.* **2008**, *80*, 6119−26.
(30) Selman, M. H.; Hemayatkar, M.; Deelder, A. M.; Wuhrer, M. *Anal. Chem.* **2011**, *83*, 2492−9.
(31) Selman, M. H.; McDonnell, L. A.; Palmblad, M.; Ruhaak, L. R.; Deelder, A. M.; Wuhrer, M. *Anal. Chem.* **2010**, *82*, 1073−81.
(32) Ceroni, A.; Maass, K.; Geyer, H.; Geyer, R.; Dell, A.; Haslam, S. M. *J. Proteome Res.* **2008**, *7*, 1650−9.
(33) Damerell, D.; Ceroni, A.; Maass, K.; Ranzinger, R.; Dell, A.; Haslam, S. M. *Biol. Chem.* **2012**, *393*, 1357−62.
(34) Ruhaak, L. R.; Steenvoorden, E.; Koeleman, C. A.; Deelder, A. M.; Wuhrer, M. *Proteomics* **2010**, *10*, 2330−6.
(35) Knezevic, A.; Bones, J.; Kracun, S. K.; Gornik, O.; Rudd, P. M.; Lauc, G. *Analyst* **2011**, *136*, 4670−3.
(36) Stumpo, K. A.; Reinhold, V. N. *J. Proteome Res.* **2010**, *9*, 4823−30.
(37) Adamczyk, B.; Struwe, W. B.; Ercan, A.; Nigrovic, P. A.; Rudd, P. M. *J. Proteome Res.* **2013**, *12*, 444−54.
(38) Townsend, R. R.; Hilliker, E.; Li, Y. T.; Laine, R. A.; Bell, W. R.; Lee, Y. C. *J. Biol. Chem.* **1982**, *257*, 9704−10.
(39) Townsend, R. R.; Heller, D. N.; Fenselau, C. C.; Lee, Y. C. *Biochemistry* **1984**, *23*, 6389−92.
(40) Maghzal, G. J.; Brennan, S. O.; George, P. M. *Thromb. Res.* **2005**, *115*, 293−9.
(41) Kornfeld, R.; Kornfeld, S. *Annu. Rev. Biochem.* **1985**, *54*, 631−64.
(42) Nairn, A. V.; York, W. S.; Harris, K.; Hall, E. M.; Pierce, J. M.; Moremen, K. W. *J. Biol. Chem.* **2008**, *283*, 17298−313.
(43) Royle, L.; Campbell, M. P.; Radcliffe, C. M.; White, D. M.; Harvey, D. J.; Abrahams, J. L.; Kim, Y. G.; Henry, G. W.; Shadick, N. A.; Weinblatt, M. E.; Lee, D. M.; Rudd, P. M.; Dwek, R. A. *Anal. Biochem.* **2008**, *376*, 1−12.
(44) Yoshikawa, H.; Hirano, A.; Arakawa, T.; Shiraki, K. *Int. J. Biol. Macromol.* **2012**, *50*, 865−71.
(45) Huffman, J. E.; Knezevic, A.; Vitart, V.; Kattla, J.; Adamczyk, B.; Novokmet, M.; Igl, W.; Pucic, M.; Zgaga, L.; Johannson, A.; Redzic, I.; Gornik, O.; Zemunik, T.; Polasek, O.; Kolcic, I.; Pehlic, M.; Koeleman, C. A.; Campbell, S.; Wild, S. H.; Hastie, N. D.; Campbell, H.; Gyllensten, U.; Wuhrer, M.; Wilson, J. F.; Hayward, C.; Rudan, I.; Rudd, P. M.; Wright, A. F.; Lauc, G. *Hum. Mol. Genet.* **2011**, *20*, 5000−11.