Highly sensitive CE-ESI-MS analysis of N-glycans from complex biological samples

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The in-depth, high-sensitivity characterization of the glycome from complex biological samples, such as biofluids and tissues, is of utmost importance in basic biological research and biomarker discovery. Major challenges often arise from the vast structural diversity of glycans in combination with limited sample amounts. Here, we present a method for the highly sensitive characterization of released N-glycans by combining a capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS) approach with linkage-specific derivatization of sialic acids and uniform cationic reducing end labelling of all glycans. This method allows the analysis of glycans at the attomole level, provides information on sialic acid isomers and enables the in-depth characterization of complex samples, even when available in minute amounts.
Lysosomal glycosylation is known as one of the most complex post-translational protein modifications and is involved in many biological processes, including cellular differentiation and antibody-receptor interactions. Characterizing the glycome of a complex biological sample can provide essential information on proteoform heterogeneity and changes in the glycan biosynthesis pathway. A major challenge posed for the glycomic analysis of biological samples is the immense complexity of the glycome. This complexity is reflected in the vast number of different structures present, including glycan isomers, and in the broad dynamic range of glycan abundances. Especially in the case of precious clinical samples, methods for the characterization of minor glycoforms and glycans originating from low-concentration glycoproteins are scarce.

Techniques currently used for the sensitive and in-depth characterization of released N-glycans involve, amongst others, capillary gel electrophoresis (CGE) coupled to laser-induced fluorescence (LIF) detection after aminopyrene trisulfonate (ATPS) labelling of the reducing ends of the glycans and nano liquid chromatography (LC)-mass spectrometry (MS) using porous graphitized carbon (PGC) as stationary phase. While the former approach shows the ability to perform a sensitive and in-depth characterization of glycan isomers in a high-throughput manner, latter approach proves particularly valuable in the case complex samples with unknown glycans are analysed, allowing their structural elucidation using negative-mode tandem mass spectrometry.

Here, we achieve high-sensitivity profiling of N-glycans by combining a capillary electrophoresis (CE) separation method with positive ion mode electrospray ionization (ESI)-MS. The sheathless interface between the CE and the ESI-MS provides low-flow (<10 nL min⁻¹) nano-ESI conditions, resulting in maximum intensities due to efficient droplet desolvation and ionization of the analytes. To enable effective electrophoretic migration of all N-glycans, sialylated species are neutralized and all species are uniformly charged by the reducing end label Girard’s reagent P (GirP). Next to neutralization, the sialic acid derivatization strategy allows to differentiate sialic acid isomers without the use of MS fragmentation, which can provide important information on biological processes, such as cancer development.

Glycoform characterization, sensitivity and repeatability. To further improve the sensitivity of the CE-ESI-MS platform, we implemented a dopant enriched nitrogen (DEN)-gas at the interface between the CE and the ESI-MS, to boost ionization of GirP-labelled glycans. Recently, we showed a substantial gain in sensitivity for the analysis of glycopeptides using DEN-gas. In the current study, the detection of the twenty most abundant GirP-labelled N-glycans in plasma with DEN-gas was compared with their detection without DEN-gas. Overall, we observed less background and an on average 3.3-fold enhancement of the signal-to-noise ratios (S/N), while the relative profiles stayed the same.

The absolute sensitivity of the overall method was assessed by analysing the glycan standards H3N4 and H5N4. A starting amount of 5 fmol of both glycans, in a sample volume of 3 µL, was sufficient for their detection and reliable relative quantification (H3N4: S/N = 49 ± 18; H5N4: S/N = 146 ± 52; Fig. 2a–c). The starting amount of 5 fmol corresponded to an absolute amount of 20 amol (43 nL corresponding to 1/250 of the sample) injected into the CE-ESI-MS system.

The performance of the fully optimized method for complex biological samples was demonstrated on TPNG, which is a sample with numerous glycoforms present in abundances that range over several orders of magnitude. In total, 167 N-glycan compositions were detected after the injection of the equivalent of 0.1 nL PNGase F treated plasma—derived from a starting amount of 0.2 µL PNGase F treated plasma—into the CE-ESI-MS setup. N-glycans were detected, and compositions were assigned, based on accurate mass and isotopic pattern. In addition, 82 glycoforms were confirmed by tandem mass spectrometry via collision-induced dissociation (CID; Fig. 3; Supplementary Fig. 6). Distinct diagnostic ions were observed in the MS/MS data of the GirP-labelled N-glycans (Supplementary Table 3), e.g. signals at m/z 656.2551⁺ and m/z 685.2605⁺ indicate B-ions indicating a full antenna loss with an amidated (a2,3-linked) or an ethyl esterified (a2,6-linked) sialic acid, respectively.
sialylated antenna, a B-ion at m/z 802.3091² was observed, indicating that fucosylation of sialylated antennae in TPNG occurs in the context of sialyl Lewis-type structures such as sialyl Lewis X₂²,²³. Furthermore, the Y-ion detected at m/z 355.1621² corresponds to the GirP label with an N-acetylglucosamine, while the ion at m/z 501.22⁰ indicates the presence of core fucosylation [GirP + N-acetylglucosamine + fucose]⁺. Bisection was identified by a Y-ion present at m/z 923.37³, or in combination with a core fucose at m/z 1069.41³.³.³

As compared with previous studies assessing the TPNG in a sialic acid linkage-specific manner, we here report a higher number of unique glycan compositions (Supplementary Data 1 and 2)²⁵,²⁶,²⁷,²⁸. The lowest abundant glycoform that could be quantified (S/N > 9) had a relative abundance of 0.007% ± 0.003% (H8N7F1S2,34 at low attomole detection level; n = 9; Supplementary Table 4 and Supplementary Fig. 7), highlighting the broad dynamic range of the platform. Only minimal (<1%) cation adduct formation was observed, resulting in simple spectra, with high sensitivity in both MS and tandem MS (Supplementary Fig. 8). While similar sensitivities and dynamic ranges were reached before with a targeted method³⁰, here we used an untargeted method, better suitable for discovery research. Furthermore, we found a very good overall method repeatability and intermediate precision, based on the relative quantification of the 118 glycoforms with a S/N > 9 (Fig. 4 and Supplementary Data 1). The preparation, analysis and relative quantification of three TPNG samples per day on three consecutive days resulted in a median RSD of the 20 most abundant N-glycans (accounting for about 80% of the N-glycans quantified) of 6.8% for the repeatability (intraday variability day 1), while the interday variability of day 1–3 showed a median RSD of 9.4%.

Conclusion. We present a highly sensitive CE-ESI-MS/(MS) platform for the in-depth analysis of N-glycans at the attomole level. Applying the method on a complex, biological sample, like the N-glycans released from total human plasma, 167 N-glycan compositions were identified, including different sialic acid linkage variants. We believe that the developed platform is of interest for the in-depth analysis of low abundant glycans in complex biological matrices. Furthermore, the glycosylation of proteins too low abundant, or samples too precious, to be measured with conventional methods becomes accessible with this method.

Methods

Materials and reagents. Ethanol (EtOH; cat. nr. 1.00983.1000), trifluoroacetic acid (TFA; cat. nr. 1.08178.0050), disodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O; cat. nr. 1197530250), potassium dihydrogen phosphate (KH₂PO₄; cat. nr. 1048730250), sodium chloride (NaCl; cat. nr. 106404) and glacial acetic acid (HAc; cat. nr. 1009631000) were purchased from Merck (Darmstadt, Germany). Recombinant peptide-N-glycosidase F (PNGase F; cat. nr. 11365177001) was acquired from Roche Diagnostics (Mannheim, Germany). Ammonium acetate (AmAc; cat. nr. A2706), 2-aminothiazole (2-AB; cat. nr. A98904), 2-picoline borane (2 PB; cat. nr. 654213), 1-hydroxysuccinimide (HOBt; cat. nr. 54802) hydrate, dimethyl sulfoxide (DMSO; cat. nr. D8418), 40% dimethyltheflamine in water (cat. nr. 426458), super-DHB (cat. nr. 50862), Nonidet P-40 substitute (NP-40; cat. nr. M158), 50% sodium hydroxide (NaOH; cat. nr. 71686), sodium dodecyl sulfate (SDS; cat. nr. L3771), and 28% ammonium hydroxide (NH₄OH; cat. nr. 221228) were purchased from Sigma Aldrich (Steinheim, Germany). 1-Ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC; cat. nr. 29810) hydrochloride was obtained from Fluorochem (Hadfield, UK). The ultrapure deionized water (MQ) used in this study was generated from a Q-Gard 2 system (Millipore, Amsterdam, Netherlands), maintained at ≥18 MΩ. LC-MS Ultra water (H₂O; cat. nr. 00232141B1BS) was purchased from Honeywell (Morris Plains, NJ) and HiPCO SuperaGradient acetonitrile (MeCN; cat. nr. 1203502) was purchased from Biosolve (Valkenswaard, Netherlands). 1-(Hydrazinocarbonylmethyl)pyrroldinum chloride (Girard’s Reagent P; GirP; cat. nr. G0030) was purchased from TCI Development Co. Ltd. (Tokyo, Japan). A standard plasma pool from healthy donors was obtained from Affinity Biologics (ViscoYm-2; Ancaster, ON, Canada; cat. nr. FRNCP0125; lot nr. 000752FCP). Two oligosaccharide standards, 3′-sialyllactose sodium salt and 6′-sialyllactose sodium salt, were obtained from Carbosynth (Compton, UK; cat. nr.; OS04397 and OS04398; lot nr. OS043971301 and OS043981501, respectively) and two glycan standards (H3N4 and H4N5; cat. nr. OS050250811) kindly provided by Ludger Ltd. (Abingdon, UK). A 10 mM phosphate-buffered saline solution (10x PBS, pH 7.2) was prepared in-house, containing 57 g L⁻¹ NaH₂PO₄·2H₂O, 5 g L⁻¹ KH₂PO₄ and 85 g L⁻¹ NaCl.
N-Glycan release. N-glycans were released from all plasma proteins (TPNG) by mixing 100 µL of plasma with 200 µL of 2% SDS followed by a 10 min incubation at 60 °C. Subsequently, a 200 µL mixture of 2.5% PBS, 2% NP-40 and 10 U PNGase F was added and the sample was incubated at 37 °C for 17 h.

Sialic acid neutralization and purification of N-glycans. Sialic acid linkage-specific derivatization by ethyl esterification was performed as described previously11. Briefly, 1 µL of released plasma N-glycans (containing the released glycans from 0.2 µL of plasma) was added to 20 µL ethyl esterification reagent (250 mM EDC and 250 mM HOBT in EtOH) and incubated for 1 h at 37 °C. Subsequently, 20 µL MeCN was added to the sample and the derivatized N-glycans were purified by cotton HILIC SPE as described previously11. Briefly, 1 µL of released plasma N-glycans was added to 20 µL dimethylamidation reagent (250 mM dimethylamine, 250 mM EDC and 500 mM HOBt and in DMSO) and incubated for 1 h at 60 °C. An additional incubation followed of 2 h at 60 °C after the addition of 8 µL 28% NH4OH. Eighty microlitres of MeCN was added to the sample and the derivatized N-glycans were purified by cotton HILIC SPE, with elution in 10 µL MQ.

Sialic acid neutralization and purification of sialyllactose. The linkage specificity of the amidation step following the ethyl esterification protocol was validated on 3'-sialyllactose and 6'-sialyllactose standards. Prior to sialic acid derivatization, the standards were labelled with 2-AB. In total, 30 µL 50 mg mL⁻¹ sialyllactose was incubated with 30 µL 24 mM NH4OH, 53.5 mg mL⁻¹ 2-AB in 7.5%/92.5% HAc/DMSO for 2 h at 60 °C. MeCN was added to the samples in a final concentration of 90% and the samples were purified by cotton HILIC SPE as described previously11, with the exception that 90% MeCN washing solutions were used instead of 85% MeCN washing solutions. Samples were eluted in 10 µL water, of which 1 µL was subjected to either ethyl esterification or EEA as described above. After incubation, 20, 24, 26 or 28 µL MeCN was added to the mixtures and the sialyllactoses were purified by cotton HILIC SPE, using 90% MeCN washing solutions and an elution in 10 µL MQ.
Permanent cationic labelling of the N-glycan reducing end. After sialic acid derivatization, either by EEA or DA, released N-glycans from plasma were labelled at the reducing end by GirP. Initial reaction conditions were as follows, 5 µL of GirP reagent (15 mM GirP in 85% EtOH, 10% HAc and 5% MQ) and incubated for 2 h at 60 °C. After incubation, 105 µL MeCN was added and cotton HILIC SPE was performed as described above, using 85% MeCN washing solutions and 10 µL elution volume. Optimization of the protocol (Supplementary Table 1) included decreasing the sample volume prior to adding 2 µL of GirP reagent (50 mM GirP in 90% EtOH and 10% HAc) and incubated for 2 h at 60 °C. After incubation, the samples were dried by vacuum concentration at 60 °C and dissolved in 10 µL MQ for CE-ESI-MS analysis.

CE-ESI-MS/MS Analysis. All experiments were performed on a 91 cm long bare-fused silica capillary (30 μm internal diameter and 150 μm outer diameter) using a CESI 8000 system (Sciex, Framingham, MA). Prior to usage, the capillary was conditioned by immersing the spray tip in MeOH while the separation and conductive lines were rinsed with MeOH at 100 psi for 10 min and 3 min, respectively. Subsequently, the tip was immersed in H2O2 and the background electrolyte (BGE; 10% HAc) to remove the UT from the outlet of the capillary. After the conditioning steps, the capillary was coated with Ultratrol on the outlet of the capillary with some small adjustments. Briefly, using 29 psi throughout, the separation line was coated by rinsing consecutively for 10 min with H2O2, 0.1 M NaOH, 0.1 M HCl, H2O and the background electrolyte (BGE; 10% HAc), followed by a final rinsing step of the conductive line for 3 min with the BGE at 100 psi. After the conditioning steps, the capillary was coated with Ultratroldynamic pre-coat LN (UT; Target Discovery, Palo Alto, CA) as described by Kohler et al. with some small adjustments. Brie...
Additional 6 min rinses with BGE were performed at 100 psi. The conductive line was rinsed with BGE for 3 min at 20 psi. Before analysis, all samples were diluted with leading electrolyte (AmAC at pH 4.0, in a final concentration of 100 mM). Injection of the samples was performed hydro-dynamically, by applying 5 psi for 60 s, corresponding to 6.8% of the total capillary volume (43 nL), unless stated otherwise. After each sample injection, a BGE post plug was injected by applying 0.5 psi for 25 s (0.3% of the capillary volume). For each analysis a constant flow was established, by applying 0.5 psi and 20 kV over the capillary with a constant temperature of 20 °C.

The CESI 8000 system was coupled to an Impact HD UHR-QqTOF-MS (Bruker Daltonics) via a sheathless CE-ESI-MS interface (Sciex) which allowed optimal alignment between the capillary spray tip and the front of the nanospray shield (Bruker Daltonics). All experiments were performed in positive-ionization mode and a stable electrospray was obtained by generating an electrical field between the CE (ground potential) and a negatively charged spray shield (between −1100 and −1300 V; for a schematic overview see Supplementary Fig. 9). For all analysis, the temperature and flow rate of the drying gas was set at 100 °C and 1.2 L min⁻¹, respectively. To minimize the in-source decay, the collision cell energy as well as the quadrupole ion energy were set at 3.0 eV and the pre-pulse storage was set at 15.0 μs. For the analysis with a dopant enriched nitrogen (DEN) gas, MeCN was used as dopant (ca. 4%, mole percent) at 0.2 bar. In addition, an in-house made polymer cone was attached onto the porous tip housing to enable the usage of the DEN-gas. Fragmentation was performed at 1.00 Hz on the three most abundant precursor ions in a range of m/z 150–2000 with a minimum intensity of 4548. Depending on the m/z values, the precursor ions were isolated with a width of 8–10 Th. The collision energies were set as a linear curve in a m/z dependent manner, ranging from 55 eV at m/z 700 to 124 eV at m/z 1800 for all charge states (1−5), applying a basic stepping mode with collision energies of 100% (80% of the time) or 50% (20% of the time).

Sensitivity assessment for the CE-ESI-MS method. Two N-glycan standards (HSN4 and HSN5) were dissolved in water and mixed in a final concentration of 3333 fmol μL⁻¹ for each glycoform. The mixture was diluted 10, 20, 100, 200, 1000, 2000 and 10,000 times and three replicates of 3 μL of each of the dilutions (10,000, 1000, 500, 100, 50, 10, 5 and 1 fmol of each glycan) were subjected to EEA and HILIC purification, and were eluted in 10 μL MQ. The replicates were dried and labelled with GirP following the optimized protocol as described above, and analysed by CE-ESI-MS.

CE-ESI-MS/(MS) data processing. Raw CE-ESI-MS data were calibrated prior to data analysis using a minimum of five signals of the identified N-glycan compositions (Supplementary Data 1) with Data Analysis 4.2 (Build 395, Bruker Daltonics). The data were manually screened for N-glycan compositions based on their exact mass, migration order and previous described structures in literature, which resulted in 167 N-glycan compositions, including differently linked sialic acids (Supplementary Data 1). Fragmentation spectra were acquired for 49% of the identified N-glycan variants of TPNG (Supplementary Fig. 6). After converting the raw files into .mzXML files, targeted data analysis was performed using an adapted version of LaCyTools v1.0.1 build 81-85. Prior to automated peak integration, all electropherograms were aligned based on 11 glycan peaks that were confirmed by tandem MS and that covered the complete migration range (34.5–39.1 min). An alignment time window of 50 s and an m/z window of 0.02 Th were used (Supplementary Figs. 10 and 11). Subsequently, for each glycan composition, the area was integrated of isotopic peaks covering at least 95% of the theoretical isotopic pattern and the background was subtracted based on the local background calculations. N-glycan compositions were included for further data analysis, when, in at least two repeated experiments per condition, their mass accuracy was between ±10 ppm for the inter- and intra-day experiments or ±20 ppm for all other analysis, their isotopic pattern did not deviate more than 20% from the theoretical isotopic pattern and their S/N was above 9. This resulted in 118 quantified N-glycan compositions in the TPNG analysis (Supplementary Data 1). The absolute abundance of the lowest abundant quantified glycoforms was estimated based on the 24 most abundant plasma glycoprotein concentrations and their glycosylation sites as reported by Clerc et al. We assumed a concentration of 0.64 mM released glycans in plasma, and a starting amount of 0.2 μL plasma. In addition, the sensitivity assessment based on the two N-glycan standards was evaluated in the condition where both HSN4 and HSN5 still passed the above stated quality criteria.

MALDI-TOF-MS analysis. After sialic acid stabilization and cotton HILIC SPE purification, the released N-glycans and the sialylactose standards were prepared for MALDI-TOF-MS analysis by applying 1 μL of the HILIC eluate on an AnchorChip 800/384 TF MALDI target (Bruker Daltonics, Bremen, Germany), together with 1 μL 5 mg mL⁻¹ super-DHB in 50% MeCN with 1 mM NaOH. The samples were left to dry by air. To assess GirP labelling efficiency, 4 μL of HILIC eluates of the GirP-labelled N-glycans, were applied on the MALDI target together with 1 μL of the super-DHB matrix. The samples were left to dry by air and re-crystallized with 0.2 μL EtOH.

MALDI-TOF-MS analysis was performed on a Bruker Daltonics UltraFlextreme, equipped with a Smartbeam-II laser and controlled by Flexcontrol 3.4 software. The instrument was calibrated with the peptide calibration standard (Bruker Daltonics) prior to analysis of the samples. MS measurements were performed in reflectron positive ion mode, using an acceleration voltage of 25 kV after 140 ns delayed extraction. Spectra were recorded between m/z 1000 and m/z 5000 (for the N-glycans) or m/z 100 and m/z 1500 (for the sialylactose standards). One-hundred 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. The laser power was adjusted to
Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The raw mass spectrometric data files that support the findings of this study are available in MassIVE in mzML and ay format, with the identifier MSV000083478 [https://doi.org/10.25345/C5061Z]35. The source data underlying Fig. 4, Supplementary Figs. 1, 2, 5, 7 and 10, and Supplementary Tables 1 and 4 are provided as a Source Data file. A reporting summary for this Article is available as a Supplementary Information file. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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G.L., N.H., P.M., S.W., M.F. and J.N. performed the experiments. G.L., N.H. and M.W. conceptually designed the work and wrote the manuscript. D.F. assisted in the experimental design. All authors read and commented on the manuscript.

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