Glycomics based biomarkers of the rate of aging : development and applications of high-throughput N-glycan analysis

Ruhaak, L.R.

Chapter 4

Optimized workflow for preparation of APTS-labeled N-glycans allowing high-throughput analysis of human plasma glycomes using 48-channel multiplexed CGE-LIF

L. Renee Ruhaak[1], René Hennig[1,2], Carolin Huhn[1,3], Matthias Borowiak[1,2], Radboud J. E. M. Dolhain[4], André M. Deelder[1], Erdmann Rapp[2], Manfred Wuhrer[1]

[1] Department of Parasitology, Biomolecular Mass Spectrometry Unit, Leiden University Medical Center, Leiden, The Netherlands

[2] Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany

[3] Central Division of Analytical Chemistry, Forschungszentrum Jülich, Jülich, Germany

[4] Department of Rheumatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

J Proteome Res. (2010) 9;6655-6664

Abstract

High-throughput methods for oligosaccharide analysis are required when searching for glycan-based biomarkers. Next to mass spectrometry-based methods which allow fast and reproducible analysis of such compounds, further separation-based techniques are needed, which allow for quantitative analysis. Here an optimized sample preparation method for N-glycan-profiling by multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF) was developed, enabling high-throughput glycosylation analysis. First, glycans are released enzymatically from denatured plasma glycoproteins. Second, glycans are labeled with APTS using 2-picoline-borane as a non-toxic and efficient reducing agent. Reaction conditions are optimized for a high labeling efficiency, short handling times, and only limited loss of sialic acids. Third, samples are subjected to hydrophilic interaction chromatography (HILIC) purification at the 96 well plate format. Subsequently, purified APTS-labeled N-glycans are analyzed by CGE-LIF using a 48-capillary DNA sequencer. The method was found to be robust and suitable for high-throughput glycan analysis. Even though the method comprises two overnight incubations, 96 samples can be analyzed with an overall labor allocation time of 2.5 hours. This method was applied to serum samples from a pregnant woman, which were sampled during first, second and third trimester of pregnancy, as well as 6 weeks, 3 months and 6 months post partum. Alterations in the glycosylation patterns were observed during pregnancy and time- after delivery.

Introduction

A large proportion of the human proteome is N-glycosylated [181]. N-glycans play a role in protein folding as well as protein solubility and are often crucial for the activity and function of the protein [1]. Therefore, analysis of protein glycosylation is gaining interest from biotechnological and pharmaceutical industry as well as clinical research. Applications in both fields require high-throughput analytical methods which allow fast and robust profiling of protein glycosylation on large sample sets.

Several strategies for fast and high-throughput analysis of N-glycans have been reported during the last years (e.g. [71;80;95;141;145]). Most of them are based on liquid chromatography (LC) or capillary electrophoresis (CE) separations which are typically performed on one sample at a time, making the analysis of multiple samples time-consuming. Run times for fast LC glycan analyses are in the range of 30 min. [80;141]. N-glycan separations by chip-CE with run times of 3 min. per sample were already demonstrated [145]; however to our knowledge the hardware is not yet commercially available. A faster approach is direct mass spectrometry using MALDI ionization, where many samples can be spotted at once for subsequent sequential analysis, with analysis times of approximately 20 sec. per sample. However, no separation of isobaric structures can be obtained, and, more importantly, quantitation using mass spectrometry is rather demanding in terms of expertise and equipment [71;131;182].

Multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF) using a DNA sequencer has been reported for the rapid analysis of 8-aminopyrene-1,3,6-trisulfonic acid (APTS)-labeled glycans [98;99;120]. CGE-LIF experiments can be performed on up to 96 samples in parallel, depending on the type of instrument. Sample clean-up after APTS labeling is currently being performed by size-exclusion chromatography. As the established protocols are rather tedious [120] or inefficient in removing the excess label [99], we were looking for efficient alternative approaches.

We here describe the development of a robust and facile high-throughput sample preparation method at the 96 well plate format for analysis of the total plasma N-glycome by CGE-LIF of APTS-labeled glycans. The efficacy of the labeling of plasma derived N-glycans with APTS was optimized using the recently reported non-toxic reducing agent 2-picoline-borane [103]. A polyacrylamide based stationary phase (Biogel P10) was used in hydrophilic interaction chromatography (HILIC) mode to purify APTS-labeled glycans. Dimethylsulfoxide has been selected as an optimal solvent for repeatable and efficient CGE electrokinetic sample injection. Peak annotation of the total plasma N-glycome was achieved by HPLC fractionation and mass spectrometric analysis of the glycans prior to APTS-labeling and CGE-LIF analysis.

Materials and Methods

Dimethylsulfoxide (DMSO), Nonidet P-40 (NP-40), triethylamine (TEA), aminopyrene-1,3,6-trisulfonic acid (APTS), sodium cyanoborohydride ($NaBH_3CN$) and 2-picoline-borane, trifluoroacetic acid (TFA), and ammonium formiate were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Sodium dodecyl sulfate (SDS) was bought from United States Biochemicals (Cleveland, OH). Glycan standards Glycan 18 (pentamannosidic N-glycan; MC0731), Glycan 27 (asialo, agalacto, bisected, core-fucosylated biantennary N-glycan; C0980), and Glycans 28 and 30 (asialo, monogalactosylated biantennary N-glycan; C0870) were purchased from Dextra Laboratories (Reading, UK). Glycan 17 (monosialylated, core-fucosylated biantennary N-glycan; M3800) was from Sigma/Aldrich. Glycan 34 (asialo, core-fucosylated biantennary N-glycan; GTP 0N-2A*F) was from TheraProteins (Oeiras, Portugal). PNGase F was obtained from Roche Diagnostics (Mannheim, Germany). Biogel P-10 was obtained from Bio-Rad (Veenendaal, The Netherlands), while glacial acetic acid, citric acid, microcrystalline cellulose and ethanol were from Merck (Darmstadt, Germany). Acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands). Sepharose Cl-4B beads (45-165 μm) were from GE Healthcare (Uppsala, Sweden). Diol-bonded silica (Chromabond Sorbenz OH) were obtained from Macherey-Nagel, Düren, Germany. Hi-Di Formamide was from Applied Biosystems/ Life Technologies (Carlsbad, CA). Human plasma for method development was ob-

tained from a healthy donor. Serum from a healthy woman with an uncomplicated pregnancy was collected in the PARA study [183] and was used for glycosylation analysis.

Preparation of oligosaccharides.

N-glycans from human plasma and serum were prepared as described previously [97]. Shortly, proteins from 10 µl of plasma or serum were denatured after addition of 20 µl 2% SDS by incubation at 60˚C for 10 min. Subsequently, 10 µl 4% NP-40 and 0.5 mU of PNGase F in 10 µl 5x PBS was added to the samples. The samples were incubated over night at 37˚C for N-glycan release.

Labeling of oligosaccharides

Labeling of oligosaccharides was performed as published [87] with slight modifications: 2 µl of N-glycan solution were mixed with 2 µl of a freshly prepared solution of label (APTS; 20 mM in 3.6 M citric acid) in a V-bottom polypropylene 96-well plate (Westburg, Leusden, The Netherlands). 2 µl aliquots of freshly prepared reducing agent solutions (different molarities of $NaBH_3CN$ or 2-picoline-borane [103] in DMSO) were added, the plate was sealed using adhesive tape and after 5 min of shaking, the samples were incubated at 37 ˚C for 16 hours. To stop the reaction, 50 µl of acetonitrile/water (80:20 v/v) were added and the samples were mixed for 5 min.

HILIC-solid phase extraction (SPE)

Free label and reducing agent were removed from the samples using HILIC-SPE. An amount of 100 µl of a 100 mg/mL suspension of cellulose, Sepharose, diol-bonded silica beads or Biogel P-10 in water/ethanol/acetonitrile (70:20:10, v/v) was applied to each well of a 0.45 µm GHP filter plate (Pall Corporation, Ann Arbor, MI). Solvent was removed by application of vacuum using a vacuum manifold (Millipore, Billerica, MA). All wells were prewashed using 5 × 200 µl water, followed by equilibration using 3 × 200 µl acetonitrile/water (80:20, v/v). The samples were loaded to the wells, and the plate was shaken for 5 min. on a shaker to enhance glycan binding. The

wells were subsequently washed using 5 × 200 µl acetonitrile/100 mM triethylamine (TEA) adjusted to pH 8.5 with acetic acid (80:20, v/v), followed by 3 × 200 µl acetonitrile/water (80:20, v/v). Washing steps were performed by addition of solutions, incubation for 30 sec., and removal of solvent by vacuum. The incubation steps were observed to result in better yields and repeatability. Water (100 µl) was applied followed by a 5 min. incubation on the shaker (to allow swelling of the Biogel P-10 particles). Thereafter, 200 µl water were added followed by a 5 min. incubation on the shaker and collection of eluates by vacuum in a 96-well V-bottom polypropylene deep well plate (Westburg, Leusden, The Netherlands). Another 200 µl water were added, followed by a 5 min. incubation on the shaker and elution into the deep well plate. The combined eluates were either analyzed immediately by CGE-LIF or stored at -20ºC until usage.

CGE-LIF using ABI-3730 DNA sequencing equipment

Two µl of N-glycan eluate were added to 60 µl of water, DMSO or Hi-Di Formamide in a PCR plate (Thermo Fischer Scientific via Westburg, Leusden, The Netherlands). Plates were sealed and turned several times for thorough mixing and subsequently centrifuged prior to analysis using an ABI-3730 DNA sequencer (Applied Biosystems) to avoid air bubbles at the bottom of the wells. The injection voltage was set at 7.5 kV, while the running voltage was 10 kV. The system was equipped with a 48 channel array with capillaries of 50 cm in length, and the capillaries were filled with POP-7 buffer (Applied Biosystems). The 3730 running buffer was obtained from Applied Biosystems. For peak annotation GeneScan 500 ROX size standard (Applied Biosystems, 2 µl of a 1:100 dilution) was added as the internal standard prior to analysis of each sample. Data was collected with a frequency of 10 Hz for 50 min.

Data processing of DNA sequencer data

Data files were converted to xml files using DataFileConverter, which is supplied by Applied Biosystems and then loaded into the in-house Matlab-based data processing tool “glyXtool” (developed at the Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg on Matlab v7.1, The Mathworks, Inc., Natick, MA).

After smoothing and background adjustment, peak heights were normalized to the sum of all peak heights in the glycan profile.

Porous graphitic carbon (PGC)- SPE of unlabeled glycan

All 96 N-glycan releases from a 96 well plate were pooled. Each sample of the plate represented an N-glycan release from 10 µl of a human plasma standard sample. The pooled N-glycans were purified by PGC according to [127]. Briefly, a PGC cartridge (Alltech, Ridderkerk, The Netherlands) was first washed using 2 x 3 ml of acetonitrile/water (80:20, v/v), then conditioned using 3 x 3 ml of water. Sample was applied in water and the cartridge was washed using 4 x 3 ml 0.1% TFA in water. N-glycans were eluted using 3 x 1 ml of acetonitrile/water (50:50, v/v) containing 0.1% TFA. The eluate was brought to dryness and reconstituted in 1 ml acetonitrile/water (80:20, v/v) prior to fractionation via HILIC-HPLC.

HILIC-HPLC separation of unlabeled glycans

N-glycans were fractionated by HILIC-HPLC (TSK amide-80, 3 µm, 150 mm x 4.6 mm inner diameter column; Tosoh Bioscience, Stuttgart, Germany). The column was run at 300 µl/min, and a binary gradient was applied using acetonitrile as solvent A, and 50 mM ammonium formiate pH 4.4 as solvent B. The following gradient conditions were applied: 0 min 30% solvent B; 5 min 30% solvent B; 50 min 50% solvent B; 51 min 80% solvent B; 54 min 80% solvent B; 55 min 30% solvent B. Fraction collection started at 35 min, and 20 sec fractions were collected in a 96-deep well plate. Fractions were dried by vacuum centrifugation.

MALDI-FTICR-MS

HILIC-HPLC fractions were reconstituted in 200 µl of water, and 1 µl of each fraction was spotted on an anchorchip MALDI target plate (Bruker Daltonics, Bremen, Germany). Spots were overlaid with 1 µl DHB (from Bruker; 20 mg/ml in 50% acetonitrile) and left to dry. MALDI-FTICR-MS experiments were performed in the positive-ion mode on a 9.4 Tesla FTICR APEX-ultra mass spectrometer equipped with a dual ESI/MALDI ion source (Apollo II), a quadrupole mass filter and a smartbeam™

laser system (Bruker Daltonics). The intermediate pressure MALDI source enables the detection of glycoconjugates containing labile monosaccharides [58]. All experiments used a laser spot size of approximately 150 µm and a laser repetition rate of 200 Hz. The quadrupole was operated in RF-only mode.

A customized pulse program was used to perform the MALDI-FTICR-MS experiments, as previously described [58]. Briefly, ions from 50 laser shots were accumulated in a hexapole and then transferred to the collision cell. This cycle was performed nine times, accumulating ions from 450 laser shots in the collision cell. The accumulated ions were then transferred to the ICR cell for a mass analysis scan. Each spectrum is the sum of eight scans. Data acquisition was performed using ApexControl 3.0.0 expert software (Bruker Daltonics), while DataAnalysis version 4.0 (Bruker Daltonics) was used for data processing.

Results

Total plasma N-glycome analysis by CGE-LIF

Total plasma N-glycome profiles were analyzed by CGE-LIF using a 48 channel DNA sequencer after N-glycan release by PNGase F, APTS-labeling, and sample purification by gel filtration or HILIC-SPE (Figure 4 1). We here describe the optimization of the sample preparation and analysis. Mild labeling conditions were chosen which to a large extent preserve the sialic acid residues. A polyacrylamide-based stationary phase allowed the fast purification of the APTS-labeled glycans at the 96 well plate format. Injection conditions were optimized to maximize injection of APTS-labeled glycans. Finally, preparative HILIC-HPLC and MALDI-FTICR-MS were applied for peak assignment. A typical electropherogram showing the obtained total plasma N-glycome profile is shown in Figure 4 2. In the migration range between 18 and 32 min. more than 30 peaks were observed, most of them baseline separated due to the large resolving power of CGE. In order to judge the optimization steps, we analyzed the electropherograms at three different levels. The signal intensity of Peak 2 was monitored as an indicator of overall glycan yield.

In order to preclude a possible bias of the sample preparation method for sialylated/ non-sialylated glycans and to register the possible loss of sialic acids, the ratio of Peak 2 (disialylated) and Peak 32 (non-sialylated counterpart) was determined (ratio Peak 2/Peak32), see Table 4 1.
We monitored the intensity of the APTS-peak in the electropherograms to judge the efficacy of sample purification in removing excess APTS.

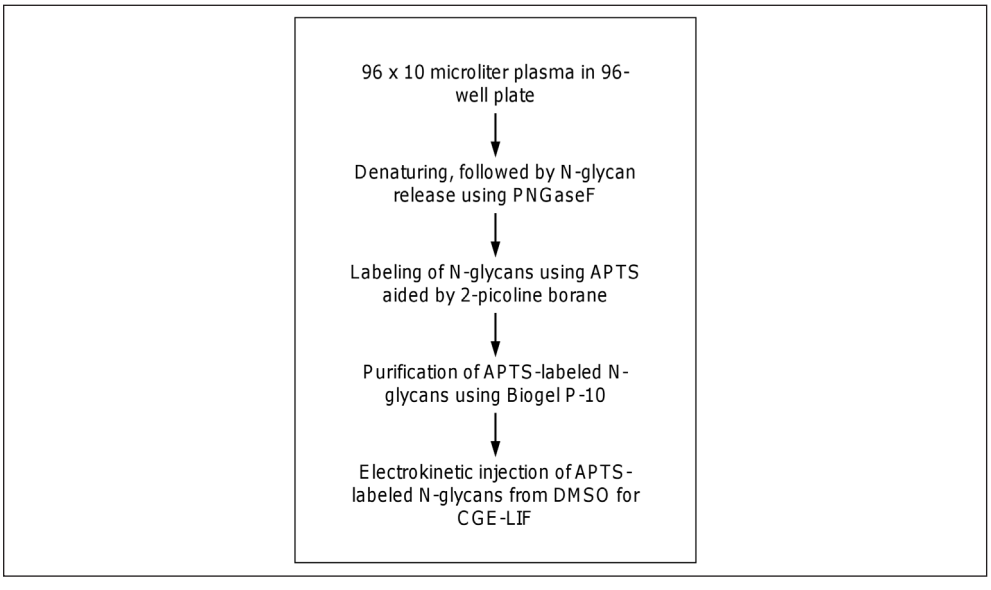
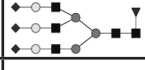
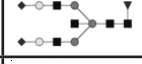
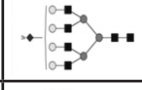
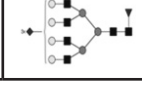


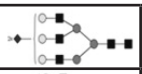
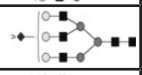
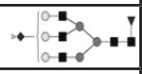
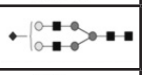
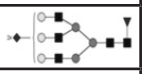
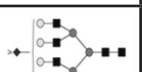
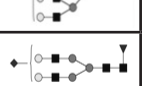
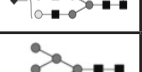
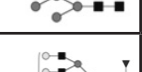
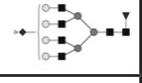
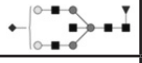
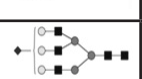
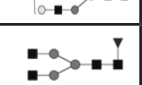
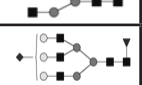
Figure 4 1. *Schematic overview of the optimized sample preparation procedure for APTS-labeled N-glycans from plasma.*

Identification of most abundant glycans.

In order to achieve an assignment of the peaks observed in the total plasma N-glycan profile (Figure 4 2), their elution positions were compared with those of APTS-labeled standard glycans of known structure (Table 4 1). This was performed after a correction for shifts in migration times: migration positions were normalized using an DNA-based (basepair) size standard, which fluoresces at a different wavelength than APTS and therefore does not interfere with the glycan signals. As

most peaks could not be assigned using glycan standards, a second strategy based on HILIC-HPLC separation of unlabeled glycans, MALDI-FTICR-MS analysis, and CGE-LIF after APTS-labeling was applied. N-glycans were released enzymatically from plasma glycoproteins, and the unlabeled glycans were preparatively separated using HILIC-HPLC. 96 Fractions were collected and one aliquot of each fraction was analyzed by MALDI-FTICR-MS in the positive mode to obtain accurate masses. A second aliquot of each fraction was labeled with APTS and analyzed using CGE-LIF according to the optimized procedure. Mass spectrometric data of the unlabeled glycans as well as elution positions in HILIC-HPLC [97] and previously published annotations of CGE-LIF profiles [99;120;148] were used to deduce the composition and structure of the glycan species in each fraction. The results are summarized in Table 4 1and Figure 4 2.

Peak No.	Peak position in base pairs	Identified by spiking in a standard oligosaccharide	Measured N-glycan mass [M + Na]+	Theoretical N-glycan mass [M + Na]+	N-Glycan composition	N-Glycan structure	Present in LC-fractions analyzed by MS	Present in LC fractions analyzed by CGE-LIF
1	172.2 (+/- 0.2)		2901.984	2902.000	$H_6N_5S_3$		66 - 71	66 - 72
2	175.8 (+/- 0.3)		2245.765	2245.772	$H_5N_4S_2$		56 - 66	56 - 67
3	177.9 (+/- 0.2)		3048.081	3048.058	$H_6N_5S_3F_1$		69 - 76	69 - 77
4	181.6 (+/- 0.3)		2245.762	2245.772	$H_5N_4S_2$		53 - 56	52 - 56
5	187.6 (+/- 0.3)		2391.822	2391.830	$H_5N_4S_2F_1$		58 - 63	58 - 63
6	191.8 (+/- 0.2)		2594.907	2594.910	$H_5N_5S_2F_1$		60 - 63	60 - 64
7	201.0 (+/- 0.3)		3267.167	3267.132	$H_7N_6S_3$		71, 73 - 76	70 - 78
8	206.6 (+/- 0.2)		3413.160	3413.190	$H_7N_6S_3F_1$		75 - 76	74 - 78

9	212.1 (+/- 0.3)		2610.892	2610.904	$H_6N_5S_2$		62 - 78	65 - 77
10	217.0 (+/- 0.3)		2610.892	2610.9044	$H_6N_5S_2$		62 - 78	62 - 71
11	223.6 (+/- 0.3)		2756.956	2756.962	$H_6N_5S_2F_1$		67 - 76	66 - 77
12	225.9 (+/- 0.4)		1954.669	1954.677	$H_5N_4S_1$		44 - 65	44 - 49
13	228.6 (+/- 0.4)		2756.956	2756.962	$H_6N_5S_2F_1$		67 - 75	66 - 77
14	233.4 (+/- 0.3)		1954.669	1954.677	$H_5N_4S_1$		44 - 65	49 - 70
15	239.7 (+/- 0.2)		1954.669	1954.677	$H_5N_4S_1$		44 - 65	53 - 56
16	248.5 (+/- 0.2)		2976.049	2976.037	$H_7N_6S_2$		71, 73, 75- 76	70 – 76, 78
17	249.5 (+/- 0.3)	X	2100.729	2100.735	$H_5N_4S_1F_1$		48 - 60	52 – 61, 63
18	250.7 (+/- 0.3)	X	1257.444	1257.423	H_5N_2		35 - 37	34 - 39
19	255.3 (+/- 0.3)		3122.097	3122.095	$H_7N_6S_2F_1$		75 - 78	73 - 78
20	256.2 (+/- 0.3)		2303.815	2303.814	$H_5N_5S_1F_1$		54 – 56, 61 - 62	52 – 57, 60 - 63,
21	257.4 (+/- 0.4)		2100.729	2100.735	$H_5N_4S_1F_1$		48 - 60	48 - 52
22	277.4 (+/- 0.4)		1419.483	1419.476	H_6N_2		44 - 48	45 - 49
23	278.3 (+/- 0.4)		2319.800	2319.809	$H_6N_5S_1$		62 - 72	61 - 71
24	281.9 (+/- 0.4)		1485.544	1485.534	$H_3N_4F_1$		25 - 29	25 - 36
25	292.8 (+/- 0.4)		2465.845	2465.867	$H_6N_5S_1F_1$		71 - 77	71 - 77
26	297.0 (+/- 0.3)		2465.845	2465.867	$H_6N_5S_1F_1$		71 - 77	71 - 77
27	297.2 (+/- 0.3)	X	1688.615	1688.613	$H_3N_5F_1$		29 - 33	29 - 34

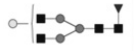
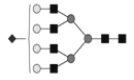
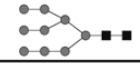
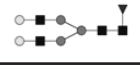
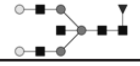
28	314.4 (+/- 0.3)	X	1647.593	1647.587	$H_4N_4F_1$		36 - 41, 44, 46- 48	34 - 45
29	318.5 (+/- 0.3)		2684.956	2684.941	$H_7N_6S_1$		73 - 76	73 - 76
30	320.8 (+/- 0.4)	X	1647.593	1647.587	$H_4N_4F_1$		36 - 41, 44, 46- 48	36 - 48
31	325.4 (+/- 0.2)		1850.645	1850.666	$H_4N_5F_1$		38, 40	37 - 42
32	327.2 (+/- 0.4)	X	1663.583	1663.581	H_5N_4		44 – 46, 50 - 60	43 - 46 50 - 60
33	351.2 (+/- 0.2)		1905.630	1905.634	H_9N_2		69 - 73	69 - 72
34	352.2 (+/- 0.4)	X	1809.644	1809.639	$H_5N_4F_1$		45 – 56, 58 - 62	45 - 56
35	361.8 (+/- 0.3)		2012.714	2012.719	$H_5N_5F_1$		47 – 50, 54 - 56	47 - 50 54 - 56

Table 4 1. *Identification and annotation of 35 N-glycan peaks from human plasma proteins. Compositions and structural schemes are given in terms of N-acetylglucosamine (square), mannose (dark circle), galactose (light circle), sialic acid (diamond) and fucose (triangle).*

Optimization of the labeling step

The conditions for labeling of oligosaccharides with APTS by reductive amination were optimized to obtain a robust, high-throughput micro-scale procedure with conservation of sialic acid residues. First, the preferred concentration of APTS for glycan labeling was determined. Plasma samples were labeled using initial concentrations of 5, 10, 20 and 40 mM APTS. The results are depicted in Figure 4 3 showing an increasing yield of APTS-labeled N-glycans with increasing APTS concentration. Because APTS is a rather expensive label, we chose to use 20 mM APTS for future experiments even though 40 mM APTS appeared to give slightly better yields of labeled glycans.

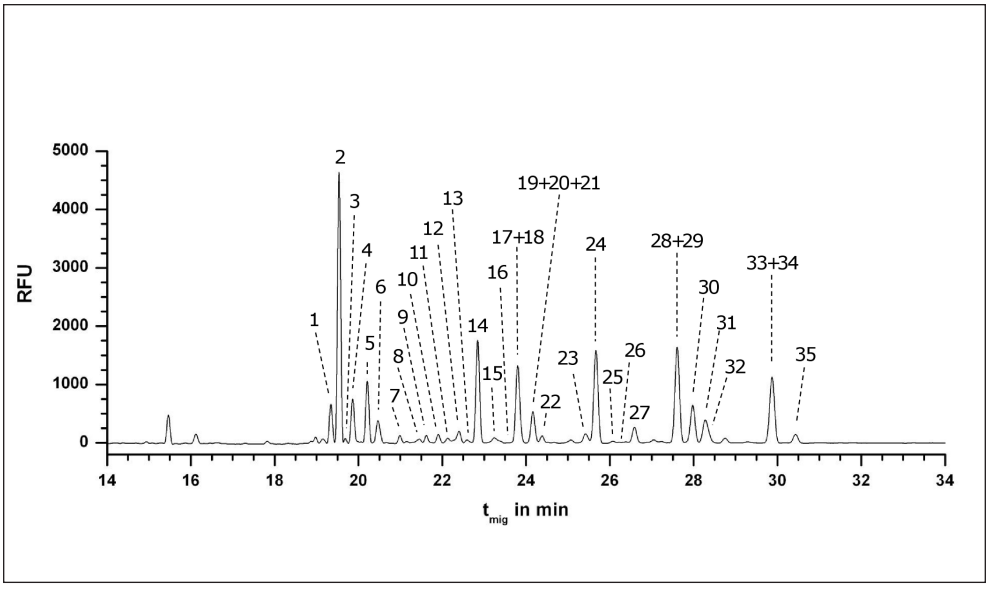


Figure 4 2. Typical electropherogram of plasma derived N-glycans labeled with APTS according to the optimized procedure. Peaks are annotated corresponding to the annotation in Table 1.

Second, the type and amount of reducing agent were optimized. Besides the widely used $NaBH_3CN$, we have applied 2-picoline-borane for the labeling of N-glycans with APTS. We recently introduced 2-picoline-borane for fluorescent labeling of oligosaccharides with 2-AA as well as 2-AB [103]. Interestingly, we could show for APTS-labeling that variation of the $NaBH_3CN$ concentration resulted in selective labeling of certain glycans, as is illustrated by the changes in ratio Peak 2/Peak 32 (ratio of non-fucosylated bisialylated Glycan 2 ($H_5N_4S_2$) over non-fucosylated non-sialylated Glycan 32 ((H5N4), see Table 4 1) in Figure 4 3. Selective labeling was, however, not observed using 1, 0.5, 0.2, 0.1 and 0.05 M 2-picoline-borane as the reducing agent (Figure 4 3). As the highest yields of labeled glycans were observed with addition of 0.2 M of 2-picoline-borane (Figure 4 3), it was decided to use 0.2 M of 2-picoline-borane for the labeling of N-glycans by APTS to minimize bias and discrimination in the labeling step.

As glycan labeling by reductive amination is acid-catalyzed, the type and concentration of acid used in the reaction may influence the yield of APTS-labeled glycans. This has indeed been reported previously, where stronger acids were associated with better yields [88]. It is also known, however, that sialic acids can be hydrolyzed from the intact glycans under acidic conditions [87]. Therefore, five different acids were applied as catalyst for the labeling of N-glycans with APTS: acetic acid (pKa = 4.74), citric acid (pKa = 3.13, 4.76, 6.4), malonic acid (pKa = 2.83, 5.13), trichloroacetic acid (TCA, pKa = 0.70) and trifluoroacetic acid (TFA, pKa = 0.30), all at a concentration of 1.2 M. It was observed that citric acid resulted in the highest yields. Because the use of citric acid also resulted in only very modest loss of sialic acids as monitored by the ratio between sialylated glycans and non-sialylated glycans (Peak 2/Peak 32), it was decided to use citric acid in the following experiments. Citric acid was then applied at four different concentrations (see Figure 4 3). It was observed that acid hydrolysis was more pronounced at higher acid concentrations. However, the yield of labeled glycans (intensity of Peak 2) also increases with increased acid concentration (Figure 4 3). A citric acid concentration of 3.6 M was chosen for futher experiments as it results in high yields and sialic acid residues are largely retained.

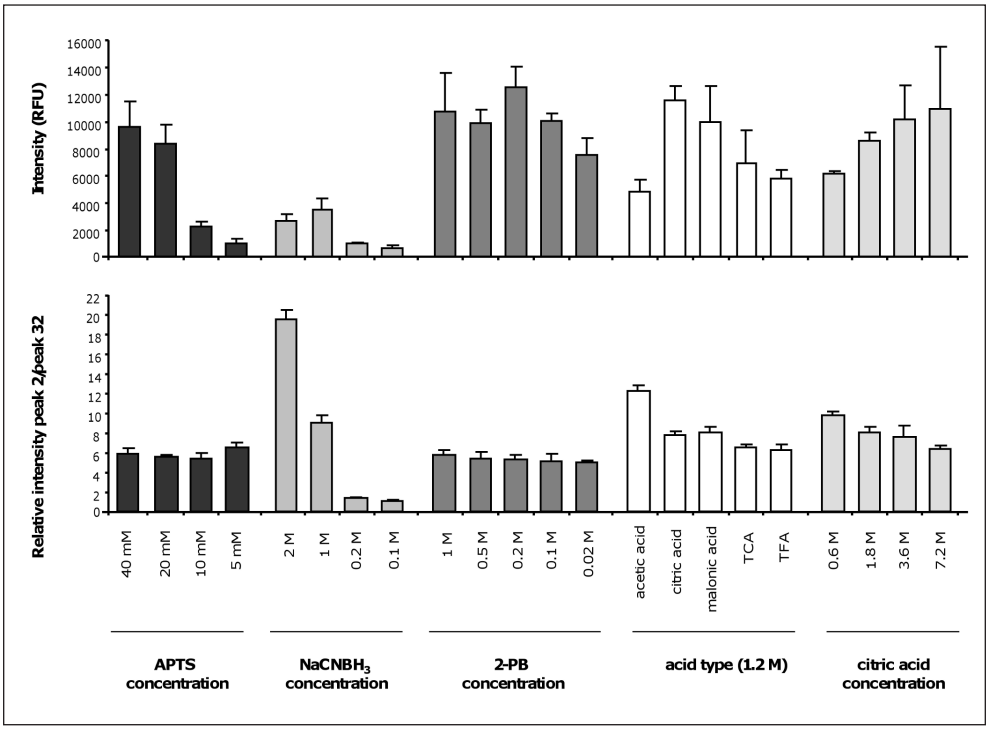


Figure 4 3. *Optimization of the labeling procedure for labeling of N-glycans with the fluorescent tag APTS. Both the absolute intensity (in RFU) of the heighest peak (Peak 2) as well as the ratio of non-fucosylated bisialylated Glycan 2 over non-fucosylated non-sialylated Glycan 32 are depicted.*

Optimization of the purification step

Fluorescent dye as well as reducing agent were employed in large excess to obtain efficient labeling. After derivatization, APTS-labeled glycans have to be purified from the reaction mixture which contains large amounts of label, reducing agent, acid, salts, SDS, NP-40 as well as protein. Previously, the use of size exclusion has been reported for purification of APTS-labeled N-glycans [99;109;120]. However, these procedures resulted in either rather tedious procedures or largely impure samples, still containing substantial amounts of the derivatization agent. Recently, we reported on a HILIC-based high-throughput purification method for 2-AA-labeled N-glycans. As the retention of labeled glycans in HILIC is mainly based on the glycan part, it was

expected that HILIC would be a good choice for purification of APTS-labeled glycans as well. Four different HILIC phases (cellulose, Sepharose, diol-bonded silica beads, and Biogel P-10, which is a polyacrylamide based phase) were employed for the purification of APTS-labeled glycans, and results were compared to those obtained by the previously published, effective but labor intensive size exclusion chromatography (SEC) procedure [120] (see Figure 4 6) in the supporting info. Clearly, the samples purified using cellulose or the polyacrylamide (Biogel P-10) phase showed electropherograms which were very similar to those obtained after SEC purification. Since cellulose is a polysaccharide phase and bleeding may occur, the polyacrylamide phase was preferred and subsequent optimization of the purification procedure was performed using this stationary phase.

As a next step, the amount of the Biogel P-10 stationary phase was optimized. For all the tested amounts of Biogel P-10 (1, 2, 3, 5, and 10 mg) the ratio sialylated/non-sialylated (ratio Peak 2/Peak 32) remained constant, and with larger amounts of stationary phase, higher peak intensities for all glycan-peaks were obtained (data not shown). An amount of 10 mg of Biogel P-10 was therefore used for further optimization. Moreover, the washing conditions were optimized. Washing using acetonitrile/water (80:20 v/v) was tested, however, large amounts of APTS could still be observed in the sample (data not shown). In order to elute APTS under washing conditions, we aimed to disrupt possible interactions of the negative charges of APTS with the stationary phase and therefore it was decided to introduce triethylamine (TEA) to the washing solution. 400 mM, 250 mM and 100 mM of TEA was added to the acetonitrile/water washing solution (80:20 v/v). As all three concentrations resulted in a similar reduction of APTS in the eluates (data not shown), the lowest TEA concentration of 100 mM TEA was used for further experiments. We then tested the effect of lowering the pH of the TEA-containing washing solution by addition of acetic acid. The pH of the washing solution containing 100 mM TEA was adjusted to 10 and 8.5 using acetic acid. The most efficient removal of APTS was observed at pH 8.5 (data not shown), and these conditions were selected for futher experiments. To examine the number of washing steps necessary to clean the derivatized glycans

from excess APTS, washing was performed three, five and seven times using 200 µl solvent. Increasing number of washing steps from three to five resulted in better purified samples, while a further increase to seven washing steps did not show further reduction of APTS (data not shown). To avoid excess salts in the eluate due to the TEA/acetic acid in the washing solvent, which would most probably interfere with the electrokinetic injection in the CGE, three additional washing steps of 200 µl acetonitrile/water (80:20, v/v) were performed. Prior to elution of the APTS-labeled glycans, a 100 µl volume of water was added to the Biogel P-10 beads. The change from acetonitrile/water (80:20, v/v) to water made the beads swell. Elution was then achieved using 2 x 200 µl water. Eluates were combined for further analysis. A third elution step was analyzed, but hardly any APTS-labeled glycans could be observed.

Sample application

Initially, samples were diluted 1:10 in 30 µl water prior to analysis by CGE-LIF. However, total signal intensities of the APTS-glycan peaks showed relative standard deviations up to 50% for replicate injections. A possible explanation for such large variation could be different salt concentration in the sample, which influences the electrokinetic injection. Therefore, in a first attempt, 1%, 4% and 10% of running buffer (void of sieving matrix) were added to the sample. Signal intensity was largely decreased already using 1% of running buffer, while the variation was still very high (data not shown). Therefore, two other options were examined: the addition of Hi-Di Formamide was tested as suggested by Applied Biosystems for sequencing analysis. Moreover, DMSO was chosen as it is a good solvent for glycans and is less volatile than aqueous solvents preventing the drying of samples during overnight runs. Interestingly, dilution of the samples in DMSO resulted in largely enhanced signal intensities, as well as much lower variation between replicates. Optimal sample application conditions were achieved by adding 2 µl of aqueous sample to 60 µl of DMSO.

Repeatability of the method

An entire 96-well plate containing aliquots of a single plasma sample from a healthy

donor was subjected to N-glycan release, APTS labeling, HILIC purification, and CGE-LIF analysis. Samples after HILIC purification were stored in the freezer and thawed on two days for second and third CGE-LIF analysis. Notably, per sample the same capillary was used for the analysis on the three different days. Data were alined, and peak heigths were determined for 12 major peaks (Figure 4 4 A).

For determination of the measurement repeatability per capillary (Figure 4 4 B), the RSD were determined by comparing the signals obtained on different days for a specific plate position (well), thereby excluding the effect of variability between capillaries. The RSDs of the specific well positions were averaged over the whole sample plate providing the intra-batch repeatability per capillary and are plotted in Figure 4 4 B. RSD values were approximately 2% for the 7 early eluting peaks and approximately 5% for the 5 late eluting peaks (Figure 4 4 B), resulting in an average RSD of 3.2% for the 12 peaks analyzed.

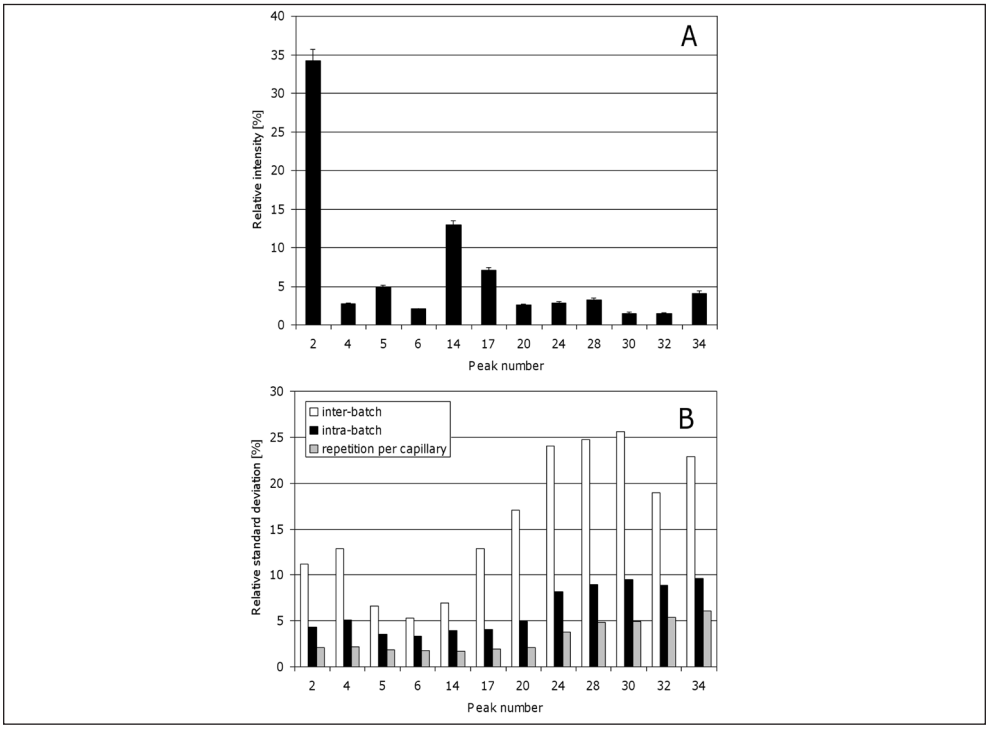


Figure 4 4. *Repeatability of plasma N-glycome analysis by CGE-LIF. (A) Average relative peak heights of the 12 major peaks are plotted together with the intra-batch standard deviation. (B) Relative standard deviations are given for the repetition per capillary and the intra-batch as well as the inter-batch repeatability test.*

To assess the overall intra-batch and inter-batch repeatability, two more 96-well plates containing replicates of the same single plasma sample were prepared on different days involving glycan release, APTS labeling, HILIC, and CGE-LIF. For intra-batch repeatablity RSDs were determined per CGE-LIF analysis for the three independent experiments, and the results were averaged. RSD values were approximately 4% for the 7 early eluting peaks and approximately 9% for the 5 late eluting peaks (Figure 4 4 B), resulting in an average RSD of 6.2% for the 12 peaks analyzed. For inter-batch repeatability RSDs were calculated per peak for the entire dataset comprising three independent experiments performed on different days. Again, early eluting peaks showed a lower inter-batch RSD than late-eluting peaks.

The average inter-batch RSD of the 12 selected peaks was found to be 15.8%.

Registration of glycosylation changes with pregnancy

In a next step we wanted to test whether the CGE-LIF method was able to show glycosylation changes associated with a biological process. Sera from a healthy woman with an uncomplicated pregnancy were taken in the 1st, 2nd and 3rd trimester of her pregnancy, as well as 6 weeks, 3 months and 6 months post partum. The total N-glycome was analyzed for all six sera. Alterations of IgG glycosylation patterns are known to be associated with different stages of pregnancy [183;184]. As expected we could observe altered glycosylation patterns in total serum N-glycans, as is illustrated in Figure 4 5. Integrals of the 13 most abundant Peaks (1, 2, 4, 5, 6, 14, 17, 20, 24, 28, 30, 32, 34) were calculated and normalized to the biantennary bisialylated Glycan 2. The glycosylation status at 6 months post partum was regarded 'normal', and therefore, the relative abundance of each glycan over the whole observation period was normalized to 100% at time point 6. Clearly, the relative levels of different glycans changed during and shortly after pregnancy. Interestingly, increases up to 170% (Glycan 4, 3rd trimester) as well as decreases up to 62% (Glycan 30, 3rd trimester) could be observed. Moreover, differences in expression time courses were observed between biantennary sialylated glycans with bisecting GlcNAc (Glycans 6 and 20) and these species lacking bisecting GlcNAc (Glycans 5 and 17): the non-bisected species were elevated during pregnancy, whilst the bisected species were not. While these data were only obtained for one pregnant woman and will therefore not be suitable to deduce general patterns of plasma glycosylation changes during pregnancy, they do clearly show that the presented high-throughput method for analysis of APTS-glycan by CGE-LIF is suitable for detecting physiological glycosylation changes.

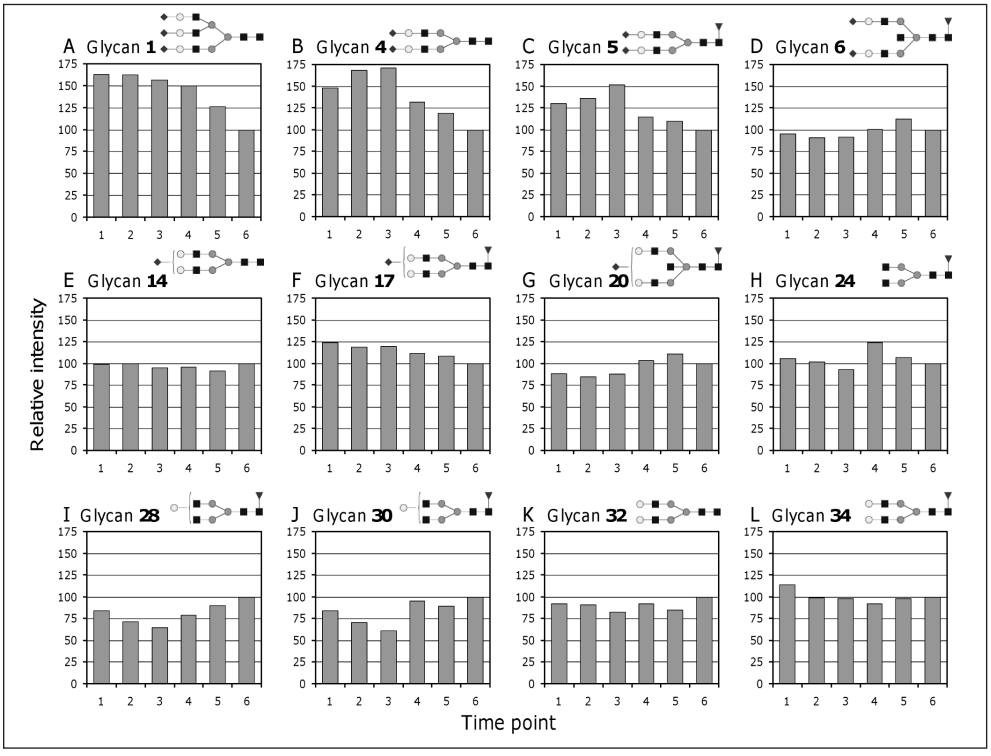


Figure 4 5. *Changes in plasma glycosylation with different stages during and after pregnancy. Integrals of 12 different N-glycan peaks are plotted relative to the bisected bisialylated Glycan 2. Integrals were determined for samples taken at six different time points: in the 1st trimester of pregnancy (1), in the 2nd trimester of pregnancy (2), in the 3rd trimester of pregnancy (3), 6 weeks post partum (4), 3 months post partum (5) and 6 months post partum (6). As the last timepoint is regarded most 'normal', these values were regarded 100%. Time series are plotted for Glycans 1 (A), 4 (B), 5 (C), 6 (D), 14 (E), 17 (F), 20 (G), 24 (H), 28 (I), 30 (J), 32 (K) and 34 (L).*

Discussion

To allow N-glycan profiling of larger sample sets, fast and robust analysis procedures are necessary. In the field of –omics analysis, glycomics is increasingly being used to generate valuable information on the glycosylation of e.g. serum IgGs or total plasma proteins. Several recent studies have shown the potential of this approach to provide novel insight in unraveling stages of healthy vs. diseased (e.g. [28;33;185]). However, clinical studies, and certainly those studies that are performed at the population level, are characterized by large sample numbers, neces-

sitating the application of fast and robust analysis procedures. Over the years, several fast procedures using direct mass spectrometry have been developed, though separation of isobaric structures is impossible using this approach. We and others recently published on fast and high-throughput sample preparation procedures for the labeling of N-glycans with 2-AA [97] or 2-AB [80], however, analysis of these samples, other than by direct mass spectrometry is still tedious, as multiplexed LC systems are not readily available.

An alternative analysis approach to MS and LC-based techniques has been published [98;99] in which a commercially available DNA-sequencer, which is commonly used in genetics departments, was used for the analysis of APTS-labeled N-glycans. In a typical 'genomics' approach, 96 samples are routinely analyzed in parallel, which indicates the potential of this method for high-throughput glycan profiling. Unfortunately, for glycan analysis applications, so far no satisfying sample preparation has been described, hampering the application of this methodology for large-scale studies. Indeed, publications so far have mainly described use of 1 to 8 channel systems [99;120].

We here describe such a high-throughput sample preparation procedure for the facile generation of APTS-labeled N-glycans from human plasma proteins. The procedure is performed at the 96-well plate level, and the whole procedure can be performed with a hands-on time of 2.5 h. The protocol is suitable for automation, and performing 2 plates in parallel reduces net labor allocation time by at least 30 min. per plate. As two overnight incubation steps are necessary, one for the release of the N-glycans and one for the labeling of the N-glycans, the total procedure takes 2.5 days. The costs for the preparation of APTS-labeled glycans at the 96-well plate level are comparable to those for the 2-AA-labeling procedure described previously [97], though APTS is much more expensive than 2-AA, the major cost factor in sample preparation being the PNGase F enzyme.

Glycan labeling was optimized to preserve sialic acid residues. While others remo-

ve sialic acid residues in order to reduce sample complexity [98;99], we chose to monitor sialylation, because plasma protein sialylation is known to be involved in determining the protein half-life with loss of sialic acids making galactose residues accessible targets for protein removal by the asialoglycoprotein receptor [186]. Moreover, hypersialylation of plasma proteins has been described in cancer [7], and IgG sialylation has been described as an anti-inflammatory signal [187;188].
The use of 2-picoline-borane in the present protocol has two major advantages compared to the conventional use of $NaBH_3CN$. First, application of 2-picoline-borane does not result in the release of HCN gas, and is thus less harmful for researchers and environment, especially when using high-throughput formats. Second, we showed that labeling of N-glycans with APTS using $NaBH_3CN$ results in selective labeling of specific glycans with a strong dependence on the concentration of reducing agent (Figure 4 2). Such biased labeling was not observed for 2-picoline-borane. Thus, the 2-picoline borane method is less susceptible to changes in the glycosylation pattern due to differences in concentration of reagents and, therefore, more robust.

We chose to test various HILIC stationary phases for purification of APTS-labeled N-glycans. Next to the polysaccharide-based stationary phases cellulose and Sepharose, we tested a diol-bonded stationary phase for HILIC-SPE. Diol-bonded stationary phases have previously been described for carbohydrate analysis by HILIC-HPLC-ESI-MS [189]. Moreover, the polyacrylamide (PAA) stationary phase Biogel P-10 was tested. While this material is commonly used for SEC, we were able to apply it in HILIC-SPE mode using solvent mixtures with 80% acetonitrile for binding and washing, and water for elution of APTS-labeled glycans. The use of a PAA-containing stationary phase for the separation of aminopyridine-labeled oligosaccharides, has been described before in HILIC-HPLC-ESI-MS [190]. Among the tested HILIC stationary phases, Biogel P-10 was chosen as it resulted in a CGE-LIF total plasma N-glycome profile which was very similar to the profile obtained after APTS-glycan purification by SEC (Figure 4 6). The APTS-glycan profile obtained after SEC purification [99;120] served as a reference and is considered to be unbiased as this

purification method (ideally) does not involve adsorption. The amount of Biogel P-10 HILIC-SPE material was varied (1-10 mg stationary phase). As expected, the overall amount of captured glycans varied, but again, we did not observe significant differences in the profiles and thus the relative intensities, demonstrating the robustness of the method for relative quantification (data not shown).

Using Biogel P-10, glycan samples cannot be contaminated with polysaccharides by bleeding of the stationary phase, as PAA is the only stationary phase in our test, which is not polysaccharide based. The HILIC-SPE procedure was optimized with regard to washing conditions. A washing solution containing 100 mM TEA/acetic acid (pH 8.5) in 80% acetonitrile was found to be largely efficient in eluting free APTS. Obviously with the positively charged TEA, ionic interaction between APTS and the stationary phase are suppressed by ion pairing. Additional washing steps with 80% acetonitrile were included in the protocol in order to get rid of salt prior to elution, thus avoiding high and differing salt content in the injection solution for CGE. APTS-labeled glycans were then eluted with water and could directly be subjected to CGE-LIF analysis.

Electrokinetic injection on the CGE-LIF system was found to be particularly efficient when a small volume of aqueous HILIC-SPE eluate was diluted with DMSO. DMSO is a good solvent for carbohydrates and, apparently, also for APTS-labeled glycans. Moreover, it shows a low volatility, and sample volumes will thus hardly change by evaporation, which is particularly relevant when long series of measurements are performed on the DNA analyzer. The high peak intensities we obtained using a high percentage of DMSO in the sample injection solution might be explained by two facts: First, DMSO is a non-protic solvent and the conductivity of the sample injection solution with a high percentage of DMSO is largely decreased. In addition, anions generally exhibit an increased pKA value in DMSO compared to water [191]. This further reduces the conductivity of the sample injection solution as weak acids like acetic acid present from the sample workup do not account for conductivity. Only the very strong acids, like the sulfonic acids of the APTS label keep their high dis-

sociation constants and thus exhibit the high electrophoretic mobility necessary for electrokinetic injection. It is interesting to note that the degree of sample dilution did not change the relative peak intensities, which shows, that there is no interference between the glycan species in quantification. This is in contrast to mass spectrometry, where quenching effects have to be taken into account.

The repeatability test showed that intra-batch RSDs for peak intensities are relatively low (6.2% on average) while the inter-batch RSDs are considerably larger (15.8%). Higher RSDs are mainly observed for the late-eluting peaks which contain non-charged glycans. The reason for this phenomenon is unknown. When large cohorts are being analyzed it may be necessary to address the inter-batch variability by performing batch-corrections per plate for statistical analysis.

A major effort was made to annotate several of the N-glycan peaks in the electropherograms, as it was impossible to couple the separation system directly with mass spectrometry. Next to the comparison of migration positions with those of standard glycans, we relied on a HILIC-HPLC fractionation of a human plasma N-glycan sample. Aliquots of each fraction were labeled with 2-AA for high-resolution MALDI analysis and with APTS for CGE-LIF. Using this combined approach, a majority of 35 peaks were annotated. For future work, we envisage a database for the CGE-LIF electropherograms of APTS-labeled N-glycans similar to Glycobase, a database for retention of AB-labeled N-glycans on HILIC-HPLC columns [135]. This is a promising project which is already started at the MPI in Magdeburg. The use of exoglycosidases, as well as more extensive, additional fractionation on a complementary separation system will however be necessary to improve the annotation. Glycans of identical composition were observed within distinct peaks (e.g. Peak 17 and Peak 21, see Table 1), demonstrating the separation of structural isomers. A similar separation was observed for the structural isomers Glycan 28 and Glycan 30, in accordance with literature.

The method was successfully applied to demonstrate changes in the total plasma

N-glycome during pregnancy. As the approach is based on the analysis of released N-glycans, no information on the identity of the glycosylated protein is obtained. Changes in the total plasma N-glycome may be caused by changes in the plasma concentration of specific glycoproteins. Alternatively, the glycosylation of an already present glycoprotein may be changed. An example for the latter is human plasma IgG, for which changes in Fc glycoforms have been described with age, pregnancy, and disease [183;184;192].

Conclusion

Labeling, purification and sample injection in CGE-LIF of APTS-derivatized human plasma N-glycans were optimized with respect to sensitivity, robustness, and minimized loss of sialic acids due to hydrolysis. Peak identification of the majority of signals, also of overlapping signals, was achieved using HILIC-HPLC fractionation and MALDI analysis of glycans. We were able to show a high robustness of the method for its use in relative quantification. The method was successfully applied in real high-throughput to real samples showing biological variation.

Overall, we here present an N-glycan profiling method which can be performed with very short handling times. The method can be used for high-throughput glycosylation analysis. An advantage over 'classical' HPLC or CE methods is that the analysis can be performed for up to 96 samples in parallel, with a very robust, easy to handle instrument, resulting in the separation of several structural isomers with high resolution.

Acknowledgements

The authors would like to thank ms. E. Steenvoorden for technical assistance. This work was supported by IOP grant IGE05007 and the Max Planck Society.

Supporting information

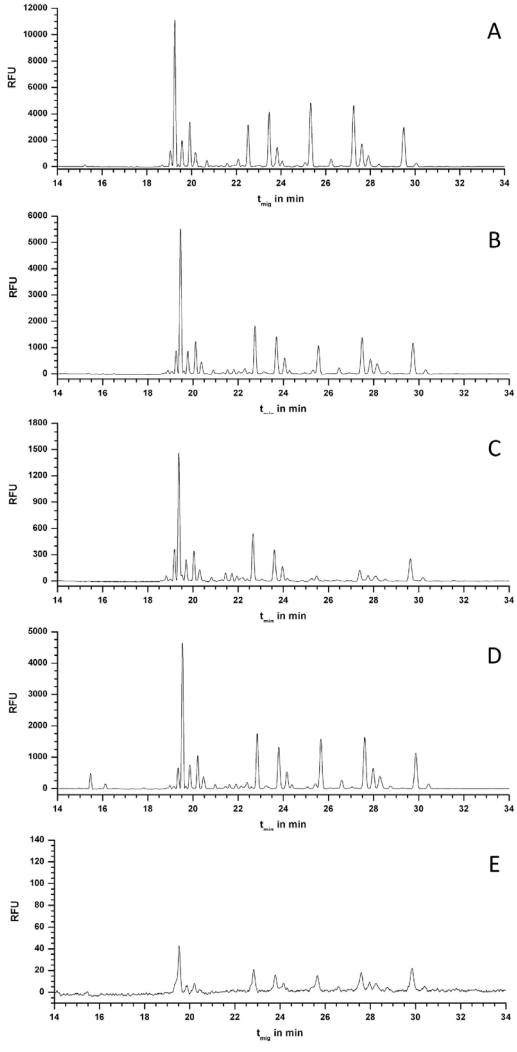


Figure 4 6. *Comparison of different stationary phases for Solid Phase Extraction of APTS-labeled N-glycans. The tedious SEC procedure using Toyopearl HW-40F resulted in electropherogram A. Electropherograms of SPE using cellulose (B), Sepharose (C), Biogel P-10, which is a polyacrylamide-based phase (D) and diol-bonded silica beads (E).*