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Chromatographic Monoliths for High-Throughput Immunoaffinity Isolation of Transferrin from Human Plasma

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THIS PAPER IS DEDICATED TO THE LOVING MEMORY OF IVANA WEYGAND-ĐURAŠEVIĆ (1952 – 2014)

Abstract: Changes in protein glycosylation are related to different diseases and have a potential as diagnostic and prognostic disease biomarkers. Transferrin (Tf) glycosylation changes are common marker for congenital disorders of glycosylation. However, biological interindividual variability of Tf *N*-glycosylation and genes involved in glycosylation regulation are not known. Therefore, high-throughput Tf isolation method and large scale glycosylation studies are needed in order to address these questions. Due to their unique chromatographic properties, the use of chromatographic monoliths enables very fast analysis cycle, thus significantly increasing sample preparation throughput. Here, we are describing characterization of novel immunoaffinity-based monolithic columns in a 96-well plate format for specific high-throughput purification of human Tf from blood plasma. We optimized the isolation and glycan preparation procedure for subsequent ultra performance liquid chromatography (UPLC) analysis of Tf *N*-glycosylation and managed to increase the sensitivity for approximately three times compared to initial experimental conditions, with very good reproducibility.

Keywords: high-throughput, immunoaffinity chromatography, monoliths, *N*-glycosylation, oriented antibody immobilization, transferrin.

INTRODUCTION

GLYCOSYLATION is a co-translational and post-translational modification of proteins by attachment of sugar moieties (glycans). It not only affects the glycoprotein structure, but also proper folding and consequently glycoprotein stability and function.[1–4] There is no direct „instruction" for glycan synthesis, hence it is the result of the amount and activity of glycosyl transferases, glycosidases, enzymes involved in sugar precursors synthesis, sugar transporters, and amount of sugar precursors.[5] Glycosylation is involved in essential cell processes including signalization, interaction and recognition of cells.[6] Therefore, it is not strange that changes in glycosylation patterns are related to congenital disorders of glycosylation,[7–9] cancer,[10] different autoimmune[11] and inflammatory diseases[12] and have a high potential as diagnostic and prognostic disease biomarkers.[13] To meet the demand of determining normal and aberrant biological glycosylation patterns of individual and total glycoproteins from body fluids and tissues in different human populations, methods for glycan analysis of large number of samples (so called high-throughput methods) are being extensively developed in recent years.[14–17]

Transferrin (Tf) is a glycoprotein that transports iron to cells and in humans has two *N*-glycosylation sites – at asparagine 432 and asparagine 630. Tf *N*-glycans are mostly of biantennary complex type with terminal *N*-acetylneuraminic (sialic) acid. Carbohydrate deficient Tf, that lacks one

or both *N*-glycans is the most common marker for congenital disorders of glycosylation.[18] Altered Tf glycosylation is also reported in hepatocellular carcinoma[19] and chronic alcohol consumption.[20,21] Although some studies on *N*-glycosylation pattern of Tf have been done to this date, biological interindividual variability of Tf *N*-glycosylation and genes involved in its regulation, to the best of our knowledge, are not known. In order to answer these questions, high-throughput glycomics methods that will enable glycosylation analysis of a large number of Tf samples are currently under extensive development.

Monolithic supports have previously been used as a tool for high-throughput immunoglobulin G (IgG) isolation from human plasma.[22] In contrast to particulate supports where molecules are transferred by diffusion, chromatographic monoliths are stationary phases cast in a single piece with highly interconnected large channels, enabling convective mass transport that results in flow independent chromatographic properties. Polymethacrylate monolithic supports have high dynamic binding capacity for large molecules and very low backpressure due to more than 60 % porosity, so they are ideal for separation of large biomolecules, such as proteins, viruses, plasmid DNA, *etc*.[23] As such, monoliths are an ideal support for high-throughput immunoaffinity applications.[24]

For Tf isolation from human plasma, an immunoaffinity purification method using convective interaction media (CIM) monolithic chromatographic support with immobilized anti-transferrin antibodies (@Tf) was performed. Oriented antibody immobilization onto CIM monoliths, whereby immobilization occurs via the antibody's carbohydrate moiety on a hydrazide or hydrazine-based supports, has been developed to enhance specificity of interaction with the antigen.[25,26] Here we show that polymethacrylate chromatographic monoliths with oriented immobilization of @Tf offer a great tool for the purification and enrichment of Tf from complex samples, such as plasma.

EXPERIMENTAL

Chemicals and Consumables

2-Aminobenzamide (2-AB), 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) buffer, 2-(*N*-morpholino)ethane sulfonic acid (MES), 2-picoline borane (2-PB), acetonitrile, dimethyl sulfoxide (DMSO), ethylene glycol, formic acid (FA), glycine, human transferrin, igepal CA630, sodium azide (NaN_3), sodium periodate ($NaIO_4$), sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glacial acetic acid and ammonia solution (φ = 25 %) were purchased from Merck (Darmstadt, Germany). GelCode Blue staining reagent was purchased from Thermo Fischer (Waltham, ME, USA). PNGase F (10 U μL^{-1}) was purchased from Promega (Madison, WI, USA). Phosphate buffered saline (PBS) was purchased from Lonza (Basel, Switzerland) or made in house: 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} Na_2HPO_4, 9.7 mmol L^{-1} KH_2PO_4, 2.2 mmol L^{-1} KCl, titrated with NaOH to pH 7.4. Sodium dodecyl sulfate (SDS) was purchased from Invitrogen (Carlsbad, CA, USA).

Buffer solutions were filtered through a 0.22 µm polyethersulfone (PES) filter (TPP, Trasadingen, Switzerland) or 0.2 µm Supor PES filters (Nalgene Thermo Fischer Scientific). Amicon Ultra-4 centrifugal filters, MWCO 10 kDa, were purchased from Merck Millipore (Billerica, MA, USA) and AcroPrep GHP filter plates from Pall Corporation (Ann Arbor, MI, USA).

Monoclonal antibodies (mAbs) for human Tf were produced at Faculty of Medicine, University of Rijeka, Rijeka, Croatia. In short, BALB / c mice were injected subcutaneously with the Tf protein (50 µg) in complete Freund's adjuvant. Two weeks later, mice were boosted with the same protein in incomplete Freund's adjuvant by injecting a two-thirds volume subcutaneously and a one-third volume intraperitoneally (i.p.). After an additional 2-week period, the sera of immunized mice were screened for antibody titers. The best responder was additionally boosted i.p. with the immunogen dissolved in PBS. Three days later, spleen cells were collected and, after lysis of red blood cells, fused with SP2/0 myeloma cells at a ratio of 1:1. The cells were seeded onto 96-well tissue culture plates in 20 % RPMI 1640 medium containing hypoxanthine, aminopterin, and thymidine for hybridoma selection. These cultures were screened for antibodies reactive against Tf by ELISA. Positive mother wells were expanded, cloned and antibodies were tested for immunoprecipitation. Finally, @Tf clone transferin.09 was expanded and secreted mAbs were purified and used for immobilization.

CIM® Protein G column (8 mL), CIMac® hydrazide column, CIM® Protein G and hydrazide 96-well plates were produced at BIA Separations, Ajdovščina, Slovenia.

Pooled plasma sample from 3 apparently healthy male adult volunteers was used for method development and all optimizations. Pooled plasma was aliquoted and frozen at –20 °C until the day of experiment. All plasma samples were centrifuged for 3 minutes at 12 100 *g*, diluted with 1 × PBS, pH 7.4, just before Tf or IgG isolation, and filtered through 0.45 µm AcroPrep GHP filter plate using vacuum manifold (around 380 mm Hg, Millipore Corporation).

Instruments Used in the Study

Centrifuge miniSpin and centrifuge model 5804 with rotor A-2-DWP (Eppendorf, Hamburg, Germany) were used for

sample processing. Analytical high performance liquid chromatography (HPLC) system, consisting of two pumps with 10 mL min^{-1} pump heads and a UV detector – Smartline (Knauer, Berlin, Germany) was used for monolithic columns characterization. Epoch BioTek microplate spectrometer (Winooski, VT, USA) was used for measuring CIMac-@Tf 96-well plate flowthrough absorbance. Mini-Protean II electrophoresis Cell (Bio-Rad, Hercules, CA, USA) and XCell SureLock™ Mini-Cell Electrophoresis System (Life Technologies, Carlsbad, CA, USA) were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Vacuum centrifuge with vacuum concentrator Savant SC210A, refrigerated vapor trap Savant RVT400 and vacuum pump OFP400 (Thermo Scientific) were used for drying of IgG and Tf eluates. MRC Basic Peristaltic Pump PP-X-575 with DG-2 head, 6 rollers and tubing: 1 mm i.d. × 1 mm wall (MRC, Cambridge, UK), vacuum manifolds (Ilmvac, Ilmenau, Germany and Millipore Corporation) and plate shaker (GFL, Burgwedel, Germany) were used during IgG and Tf isolation, and subsequent sample preparation for glycan analysis. Acquity ultra performance liquid chromatography (UPLC) instrument (Waters, Milford, MA, USA) consisting of a quaternary solvent manager, sample manager and a fluorescence detector was used for glycan analysis.

Preparation of Columns with Immobilized @Tf

Immunoaffinity chromatographic monoliths were prepared based on the CIMac® hydrazide columns with column volumes of 0.1 mL and average pore size diameter of 1.4 µm. @Tf mAbs were first purified from serum free supernatant using CIM® Protein G 8 mL column. The supernatant was diluted with PBS (Lonza), ψ(supernatant, PBS) = 1:1, loaded onto the column at flow rate of 8 mL min^{-1} and eluted with 3 column volumes of 100 mmol L^{-1} glycine, pH 2.7, at 8 mL min^{-1}. pH of the elution fraction was adjusted to 7.0 with 1 mol L^{-1} Tris buffer, pH 9.0.

The mAbs were concentrated to 2 mg mL^{-1} and buffer was exchanged to 10 mmol L^{-1} phosphate, 100 mmol L^{-1} NaCl buffer, pH 7.2, using Amicon Ultra-4 centrifugal filter, MWCO 10 kDa. The dissolved mAb stock was mixed with a solution of 20 mmol L^{-1} $NaIO_4$ in phosphate buffer at ratio of 1:1 (*v*/*v*) and thermostated for 30 min at 25 °C to achieve the oxidation of glycosylated moieties of the mAb to aldehyde functional groups. The reaction was quenched by addition of ethylene glycol (25 µL of ethylene glycol per mL of the mAb solution) to reaction mixture. Further, mAb solution was diluted twenty-fold with 50 mmol L^{-1} MES buffer, pH 5.2. The immobilization solution containing 1 mg of dissolved mAbs was pumped through a preconditioned CIMac® hydrazide column at 0.5 mL min^{-1}. The column was closed with blind stoppers and thermostated for 15 hours at 25 °C. Prepared column was finally washed with 50 mmol L^{-1} MES, 1 mol L^{-1} NaCl solution, pH 5.2 and stored in PBS, pH 7.2, 0.2 g L^{-1} NaN_3.

Preparation of 96-Well Plate with Immobilized @Tf

@Tf mAb for 96-well plates was purified and treated with $NaIO_4$ as described above. Subsequently, 0.5 mg of the mAb per well of the CIM® hydrazide plate (200 µL monolith with average pore size diameter of 2.1 µm) was loaded using a vacuum manifold (Ilmvac). The flowthrough solution was re-pumped through the plate 10 times during 24 h cycle. Finally, each well of the plate was washed with 50 mmol L^{-1} MES, 1 mol L^{-1} NaCl solution, pH 5.2 and stored in PBS, pH 7.2, 0.2 g L^{-1} NaN_3.

Specificity of Immunoaffinity Monoliths for Tf

Plasma sample was prepared as described in Chemicals and consumables. Selectivity of prepared @Tf immunoaffinity monoliths was tested by bind-elute mode using the analytical HPLC system and EuroChrom 2000 software (Knauer). In short, plasma sample diluted with PBS, ψ(sample, PBS) = 1:9, was injected onto the CIMac-@Tf column at 1.0 mL min^{-1}. The column was washed with PBS and Tf was eluted with 0.1 mol L^{-1} formic acid, pH 2.4. Flowthrough and elution fractions were collected when UV_{280} absorbance peak was detected, and analyzed by SDS-PAGE. Undiluted elution fractions were loaded onto 4–20 % gradient gels under reducing conditions according to the manufacturer's instructions (Bio-Rad). Gels were run at 200 V for 35 min using a discontinuous Tris-glycine buffering system. Protein bands were visualized by GelCode Blue staining reagent.

Determination of Dynamic Binding Capacity for Tf

Dynamic binding capacity (DBC) was determined for CIMac-@Tf column with frontal analysis experiments using the analytical HPLC system. Tf was dissolved in PBS (γ = 0.05 mg mL^{-1}), and loaded on the CIMac-@Tf column at 1.0 mL min^{-1} until the point of breakthrough. The dynamic binding capacity at 50 % breakthrough (DBC_{50}) was calculated from $t_{50\ \%}$ at which UV_{280} was half-maximal according to the [Eq. (1)]:

$$DBC_{50} = \frac{(t_{50\ \%} \times \Phi - V_d) \times \gamma_0}{V_c} \quad (1)$$

where Φ represents the flow rate (mL min^{-1}), $t_{50\ \%}$ is the time when the absorbance reached the 50 % of the breakthrough curve, V_d is the dead volume of the system (mL),

γ_0 is the initial protein concentration (mg mL^{-1}) and V_c is the total monolith volume.

The DBC for CIMac-@Tf 96-well plate was determined using a vacuum manifold instead of the HPLC system to pump the solutions through the monolith. Aliquotes of 500 µL Tf in PBS (γ = 0.1 mg mL^{-1}) were repeatedly added and plate flowthrough absorbance measured. The elution was performed by the addition of 1 mL of 0.1 mol L^{-1} formic acid to each well. Elution fractions were collected and absorbance at 280 nm was measured.

IgG Removal from Plasma Samples

IgG was removed from blood plasma samples by CIM® Protein G 96-well plate, using vacuum manifold (Millipore Corporation). All steps during the isolation procedure were performed at around 380 mm Hg, except for plasma sample application and IgG elution (around 200 mm Hg). Protein G plate was washed with 2 mL of ultra-pure water (18 MΩ cm at 25 °C), 2 mL of 1 × PBS, pH 7.4, and 1 mL of 0.1 mol L^{-1} formic acid; neutralized with 2 mL of 10 × PBS; and equilibrated with 4 mL of 1 × PBS, pH 7.4.

Plasma samples, ψ(sample, 1 × PBS, pH 7.4) = 1:7, were applied to the CIM® Protein G plate, and flowthrough was collected for subsequent Tf isolation. Unbound proteins were washed away with 3×2 mL of 1 × PBS, pH 7.4. Bound IgG was eluted with 1 mL of 0.1 mol L^{-1} formic acid and neutralized with 1 mol L^{-1} ammonium hydrogencarbonate to pH 7.0.

The CIM® Protein G plate was washed with 1 mL of 0.1 mol L^{-1} formic acid, 2 mL of 10 × PBS, 4 mL of 1 × PBS, pH 7.4, 2 mL of storage buffer (ethanol φ = 20 %, 20 mmol L^{-1} Tris, 0.1 mol L^{-1} NaCl, titrated with HCl to pH 7.4), and stored at 4 °C.

Transferrin Isolation from Blood Plasma

Tf was isolated from 70 or 100 µL of human plasma pool, ψ(sample, 1 × PBS, pH 7.4) = 1:7 or 1:9 using peristaltic pump (flow rate of 0.5 mL min^{-1} or 1 mL min^{-1}) or vacuum manifold, in case when CIMac-@Tf column or CIMac-@Tf 96-well plate were used for isolation, respectively.

The CIMac-@Tf column was washed with 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0 or 3.5 [pH is adjusted with ammonia solution (φ = 25 %), fraction F1] and 2 mL of 1 × PBS, pH 7.4. Diluted plasma sample was applied to the column and column was washed with 6 mL of 1 × PBS, pH 7.4 (fractions W1–W6). Tf was eluted with 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0 or 3.5 (fractions of 0.5 mL were collected, E1–E2). The CIMac-@Tf column was additionally washed with 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0 or 3.5 (fraction E3), 1 mL of 0.1 mol L^{-1} formic acid, pH 2.5 (fractions of 0.5 mL were collected, E4–E5), 3 mL of 1 × PBS, pH 7.4 (fractions P1–P3) and 2 mL of storage buffer (1 × PBS, pH 7.4, 0.2 g L^{-1} NaN_3). All acidic fractions were neutralized with 1 mol L^{-1} ammonium hydrogencarbonate to pH 7.0. Each elution or washing fraction (300 µL or 500 µL) was dried in a vacuum centrifuge for subsequent glycan analysis.

CIMac-@Tf 96-well plate was used in a similar way, with following differences: plate was equilibrated with 4 mL of 1 × PBS, pH 7.4 (instead of 2 mL) before sample application, Tf was eluted from the plate with 0.1 mol L^{-1} formic acid, pH 3.0, and plate was washed with 4 mL of 1 × PBS, pH 7.4 (instead of 3 mL), before washing with storage buffer.

The CIMac-@Tf column and the CIMac-@Tf 96-well plate were stored at 4 °C. All sample fractions were stored at –20 °C.

Elution fractions were analyzed by SDS-PAGE using 4–12 % Bis-Tris gradient gels (1.0 mm thickness) under reducing conditions according to the manufacturer's instructions (Life Technologies). The gels were run at 200 V for 35 min using a MES SDS buffering system. Protein bands were visualized by GelCode Blue staining reagent.

Desalting of Tf Samples Before Deglycosylation

Dried Tf eluates were desalted by adding 1 mL of cold methanol (previously cooled down to –20 °C). Samples were resuspended with a pipette and centrifuged for 15 minutes at 2200 *g* with adhesive seal (Eppendorf centrifuge, model 5804). Supernatant (970 µL) was carefully removed and procedure was repeated. Remaining methanol was removed by drying desalted samples in the vacuum centrifuge.

N-Glycan Release and Labeling

The whole procedure was done in a 96-well plate manner and ultra-pure water was used throughout. Dried Tf eluates were denatured with 30 µL of SDS (γ = 13.3 g L^{-1}) and by incubation at 65 °C for 10 min. After cooling down to room temperature for 30 min, 10 µL of Igepal CA-630 (φ = 4 %) was added and mixture was shaken for 15 min on a plate shaker. *N*-glycans were released after addition of 1.2 U of PNGase F in 10 µL of 5 × PBS by incubation at 37 °C for 18 hours. Released *N*-glycans were labeled with 2-AB. The labeling mixture was freshly prepared by dissolving 2-AB (final γ = 19.2 mg mL^{-1}) and 2-PB (final γ = 44.8 mg mL^{-1}) in the mixture of DMSO and glacial acetic acid, ψ(DMSO, CH_3COOH) = 7:3. To each of the *N*-glycan samples 25 µL of the labeling mixture was added and the plate was sealed using an adhesive seal. Mixing was achieved by 10 min shaking, followed by 2 hour incubation at 65 °C.

Clean-Up of 2-AB Labeled Glycans

Free 2-AB label, excess of reagents and proteins were removed from the samples after *N*-glycan release and

labeling using hydrophilic interaction liquid chromatography solid phase extraction (HILIC-SPE). After cooling down to room temperature for 30 min, 700 µL of acetonitrile (previously cooled down to 4 °C) was added to each sample. The clean-up procedure was performed on a hydrophilic 0.2 µm AcroPrep GHP filter plate. Solvent was removed by vacuum manifold at around 25 mm Hg. All wells were prewashed with 200 µL of ethanol in water (φ = 70 %), 200 µL of ultra-pure water and 200 µL of acetonitrile in water (φ = 96 %, previously cooled down to 4 °C). The samples diluted with cold acetonitrile were loaded to the wells, and after short incubation subsequently washed with 5 × 200 µL of acetonitrile in water (φ = 96 %, previously cooled down to 4 °C). Glycans were eluted with 2 × 90 µL of ultra-pure water after 15 min shaking at room temperature and combined eluates were stored at –20 °C until the UPLC analysis.

Glycan Analysis by Ultra Performance Liquid Chromatography

Fluorescently labeled and purified *N*-glycans were separated by HILIC-UPLC and detected using excitation and emission wavelengths of 250 and 428 nm, respectively. The instrument was under the control of Empower 3 software, build 3471 (Waters). The 2-AB labeled *N*-glycans were separated on a Waters BEH Glycan chromatography column, 150×2.1 mm i.d., 1.7 µm BEH particles, with 100 mmol L^{-1} ammonium formate, pH 4.4, as solvent A and acetonitrile as solvent B. The 2-AB labeled glycan samples were prepared in acetonitrile, ψ(sample, acetonitrile) = 25:75, and analyzed in linear gradient of 30–47 % solvent A at flow rate of 0.56 mL min^{-1} in a 23 min analytical run. Samples were maintained at 10 °C before injection onto the column, and the column temperature was 25 °C. The HILIC-UPLC system was calibrated using an external standard of hydrolyzed and 2-AB labeled glucose oligomers (dextran ladder) according to which the retention times for the individual chromatographic peaks (representing the 2-AB labeled glycan) were converted to glucose units. Data processing was performed using an automatic processing method with a traditional integration algorithm. Furthermore, each chromatogram was manually corrected to maintain the same intervals of integration for all the samples. The samples were all separated into 35 peaks and the amount of glycans in each chromatographic peak was expressed as percentage of total integrated area (% Area).

RESULTS AND DISCUSSION

Chromatographic Characterization of Monolithic Columns and a 96-Well Plate for Tf Isolation

The CIMac-@Tf columns were successfully prepared according to procedure developed for immobilization of polyclonal antibody specific for human serum albumin[27] using a mAb specific for transferrin (@Tf) and a hydrazide column, as described in the paragraph Preparation of columns with immobilized @Tf. After antibody immobilization, we confirmed that the column is specific for Tf. Since the final application for the prepared columns is isolation of the protein from plasma sample, we evaluated the column's specificity using a HPLC system and 1 mL of 10 times diluted plasma sample in bind/elute mode. In order to test the column efficiency at high-throughput conditions, we used a high flow rate of 1.0 mL min^{-1}. The bound proteins were eluted using 0.1 mol L^{-1} of formic acid, pH 2.4 (Figure 1). The analysis of collected fractions by SDS-PAGE showed that the purity of the elution fraction was above 95 % (Figure 1, insert). The Tf was eluted from the column in 500 µL of elution buffer, corresponding to 5 column volumes. However, the CIMac-@Tf column started deteriorating with the consecutive bind-elute runs and therefore the optimization of elution buffer was performed. The initial formic acid elution buffer was partially neutralized with NaOH to pH values of 3.5 and 3.0. A large elution peak was observed at pH 3.5, and after changing the medium to pH 3.0 an additional elution peak was observed, but it comprised less than 0.5 % of total area (results not shown). Although total elution of Tf was achieved even with 0.1 mol L^{-1} formic acid, pH 3.5, pH 3.0 was selected for further experiments in order to avoid possible cross-contamination between separate isolations. Additionally, Na^+ ions are incompatible with downstream analysis of Tf glycosylation and thus the final elution buffer was pH adjusted using NH_3. After the initial protocol was established, the column was tested with 5 consecutive

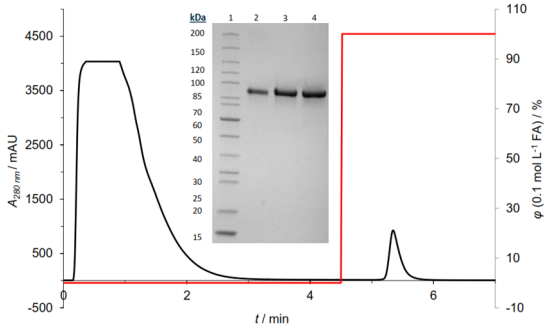

Figure 1. Black line: typical HPLC-UV profile of plasma loading and elution from CIMac-@Tf column. Red line: elution of bound proteins with 0.1 mol L^{-1} formic acid, pH 2.4. Insert: SDS-PAGE gel of Tf standard and Tf isolated from human plasma in the 1st and 4th consecutive isolations on the same column. Lanes: (1) 10–200 kDa molecular mass standard (Fermentas Life Sciences, Burlington, Canada); (2) standard human Tf (0.1 mg mL^{-1}); (3) Tf elution from the 1st isolation; (4) Tf elution from the 4th isolation.

bind-elute experiments and no deterioration of the column was observed anymore.

To confirm the stability of the CIMac-@Tf column and complete elution of bound Tf, fractions collected during Tf isolation have been analyzed by SDS-PAGE (not shown). Additionally, HILIC-UPLC glycan analysis was performed for all fractions since it can detect antibody leakage in the washing steps after column storage or during Tf isolation procedure (Figure 2). There was no detectable antibody leakage during the washing step before sample application (fraction F1, Figure 2) and the majority of the bound Tf eluted from the column in the first 0.5 mL of 0.1 mol L^{-1} formic acid, pH 3.0 (fraction E1, Figure 2).

Initial amount of Tf is important for reproducible glycosylation analysis by HILIC-UPLC and around 300 µg of purified Tf is required from a single capture step from blood plasma. Therefore, the binding capacity for pure Tf dissolved in PBS buffer was determined for prototype CIMac-@Tf columns. The DBC_{50} values were between 3.5 and 3.9 mg of Tf per mL of monolithic support, enabling isolation of more than 300 µg of Tf in a single run using 0.1 mL CIMac-@Tf column.

High-throughput method development is essential for fast analysis of Tf glycosylation in large number of samples. For parallel isolation of Tf from 96 plasma samples we prepared a 96-well plate with immobilized @Tf as described in Experimental section. Since large-pores monoliths have higher permeability and consequently decreased possibility of clogging and higher throughput, we used 2.1 µm average pore size diameter monoliths for CIMac-@Tf 96 well plate preparation, compared to 1.4 µm average pore size diameter monoliths used for CIMac-@Tf columns. On the other hand, large pores decrease the surface area of the monolith and result in decreased binding capacity for antigen,[28] so monoliths of larger volume (200 µL) were used for CIMac-@Tf 96-well plate, compared to the monoliths used for the column format (100 µL). After @Tf immobilization the CIMac-@Tf 96-well plate was characterized for pure human Tf binding capacity with the same reagents as with the CIMac-@Tf columns. There was no difference in absorbance between effluent fractions and PBS buffer at 280 nm (before each column was saturated), proving complete binding of Tf and no leakage of @Tf. After complete Tf saturation of the immunoaffinity sorbent, the plate was washed with PBS buffer, followed by protein elution and quantification. The average amount of eluted Tf calculated from absorbance reading at 280 nm was 300 µg per well with relative standard deviation (RSD) of 9.1 % for the whole plate, what is comparable with commercially available CIM® protein G or protein A 96-well plates. The Tf elution capacity was within previously determined requirements for amount of purified Tf and newly prepared CIMac-@Tf 96-well plate could be used for larger population studies.

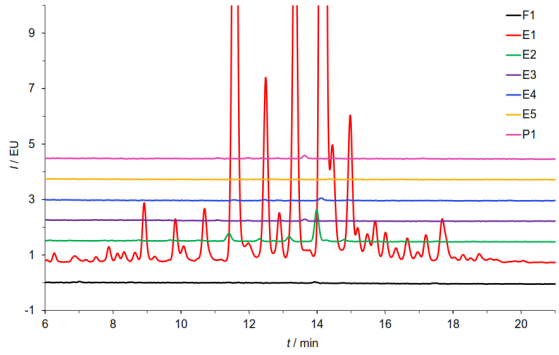

Figure 2. Glycosylation profiles of fractions collected during Tf isolation from human blood plasma on CIMac-@Tf column. F1 – wash with 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0 (pH adjusted with ammonia solution, φ = 25 %) before plasma sample application; E1, E2 – Tf elution fractions with 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0 (0.5 mL each); E3 – additional wash with 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0; E4, E5 – additional wash with 1 mL of 0.1 mol L^{-1} formic acid, pH 2.5 (0.5 mL each); P1 – wash with 1 mL of 1x PBS, pH 7.4. EU – emission units.

Optimization of Tf Isolation and Glycan Preparation

Tf *N*-glycome typically contains only 5–6 high abundant (> 2 % of the total *N*-glycome each) *N*-glycans (Figure 3). Since low abundant peaks generally have higher coefficients of variation, we tried to improve the sensitivity of quantification by optimizing the conditions for Tf isolation and glycan preparation before the UPLC analysis. Originally, after isolation from blood plasma Tf was eluted from CIMac-@Tf 96-well plate in 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0 and 300 µL of eluate was dried and

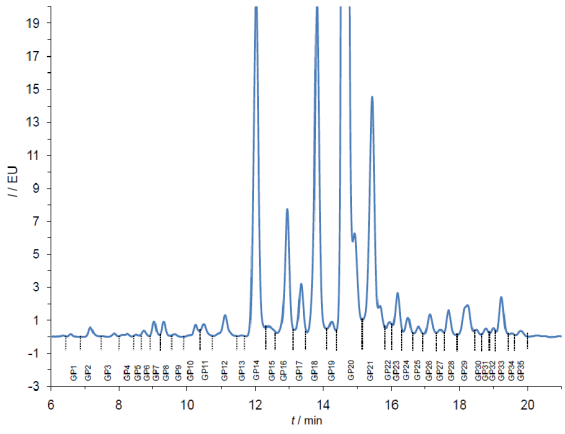

Figure 3. Representative HILIC-UPLC chromatogram of Tf *N*-glycan peaks (GP1-GP35). EU – emission units.

deglycosylated as described in Experimental section. After clean-up, 2-AB labeled glycans are typically eluted from hydrophilic GHP filter plate in 2 × 90 µL of ultra-pure water. We tested different elution volumes during transferrin isolation (1 mL and 0.7 mL) and the volume of transferrin eluate used for deglycosylation (300 µL and 500 µL). Since an extensive amount of salts in protein sample can affect reaction yields, we desalted isolated transferrin with methanol. In order to get even more concentrated sample, we tested different conditions for glycan elution from GHP filter plate (Table 1). Each experiment was performed in four replicates with the same plasma sample.

Table 1. Optimization of transferrin isolation and glycan preparation conditions.

$V_{elution}$[(a)] / mL	V_{dried}[(b)] / µL	Desalted with MeOH	$V_{glycan\ eluate}$[(c)] / µL
1	300	YES	2 × 25
			1 × 50
			2 × 50
			2 × 90
		NO	2 × 25
			1 × 50
			2 × 50
			2 × 90
	500	YES	2 × 25
			1 × 50
			2 × 50
			2 × 90
		NO	2 × 25
			1 × 50
			2 × 50
			2 × 90
0.7	300	YES	2 × 25
			1 × 50
			2 × 50
			2 × 90
		NO	2 × 25
			1 × 50
			2 × 50
			2 × 90

(a) $V_{elution}$ – volume of elution fraction during Tf isolation.

(b) V_{dried} – volume of Tf eluate used for deglycosylation.

(c) $V_{glycan\ eluate}$ – volume of ultra-pure water used for glycan elution from GHP plate after clean-up procedure.

Each experiment was performed in four replicates from the same initial plasma sample. Grey shaded combinations of experimental conditions are further tested on larger number of samples (see Table 2).

Several tested combinations of preparation conditions (volume of elution fraction during Tf isolation, volume of Tf eluate used for deglycosylation, desalting with methanol, and volume of ultra-pure water used for glycan elution from GHP plate after clean-up procedure) gave significantly improved intensities of glycan peaks in HILIC-UPLC chromatogram (around 250 EU) compared to previously used standard preparation conditions described in the previous paragraph (around 50 EU, dark grey in Figure 4). Desalting with methanol was shown to be somewhat challenging to perform, and could lead to sample losses in methanol removing steps. Due to these reasons desalting with methanol was excluded from the final protocol for Tf glycan analysis. However, in the absence of methanol desalting step, we observed that for Tf samples that contained a higher amount of salts (ammonium formate) and proteins, we had to add more than 100 µL of ultra-pure water for the glycan elution in order to obtain sufficient glycans recovery from GHP filter plate after clean-up procedure. In contrast to that, all samples that have previously been desalted with methanol, showed very good glycan recovery even when only 50 µL of ultra-pure water was used for glycan elution from GHP filter plate. Also, by increasing the volume of ultra-pure water for glycan elution, signal intesities were getting lower due to decreasing glycan concentration in eluates, which is expected, but is only noticed in samples that have been desalted with methanol, and in samples that have not been desalted with methanol but contained the lowest amount of salts and proteins (Figure 4). This phenomenon has already been noticed in HILIC chromatography and is explained by suppresion of negatively charged sialic acids repulsion by presence of salts in mobile phase, which in result allows tighter binding of sialylated glycans to the water-enriched solvent layer on the surface of the stationary phase.[29] Additionaly, we noticed that chromatographic profiles of Tf glycans were different depending whether samples were desalted with methanol before deglycosylation or not. Since transferrin contains mostly sialylated *N*-glycans, this mechanism obviously plays a significant role in glycan clean-up step.

In order to be able to assess the variability of the method, following experimental conditions were further tested with larger number of samples (n = 24 for each): (i) transferrin elution from monolithic support with 1 mL of formic acid, drying and deglycosylation of 300 µL of eluate, glycan elution in 2 × 25 µL of ultra-pure water, (ii) transferrin elution from monolithic support with 1 mL of formic acid, drying and deglycosylation of 500 µL of eluate, glycan elution in 2 × 50 µL of ultra-pure water, (iii) transferrin elution from monolithic support with 0.7 mL of formic acid, drying and deglycosylation of 300 µL of eluate, glycan elution in 2 × 50 µL of ultra-pure water (Table 1).

Transferrin elution from monolithic support with 0.7 mL of formic acid, followed by drying and deglycosylation of 300 µL of eluate and glycan elution in 2 × 50 µL of ultra-pure water, gave the most reproducible results. The peak percent area CVs were < 5 % for 12 peaks, 5–10 % for 13 peaks, 10–15 % for 4 peaks, and > 15 % for 6 peaks, compared to the other two tested combinations where only 4 out of 35 glycan peaks had CVs < 5 % (see Table 2).

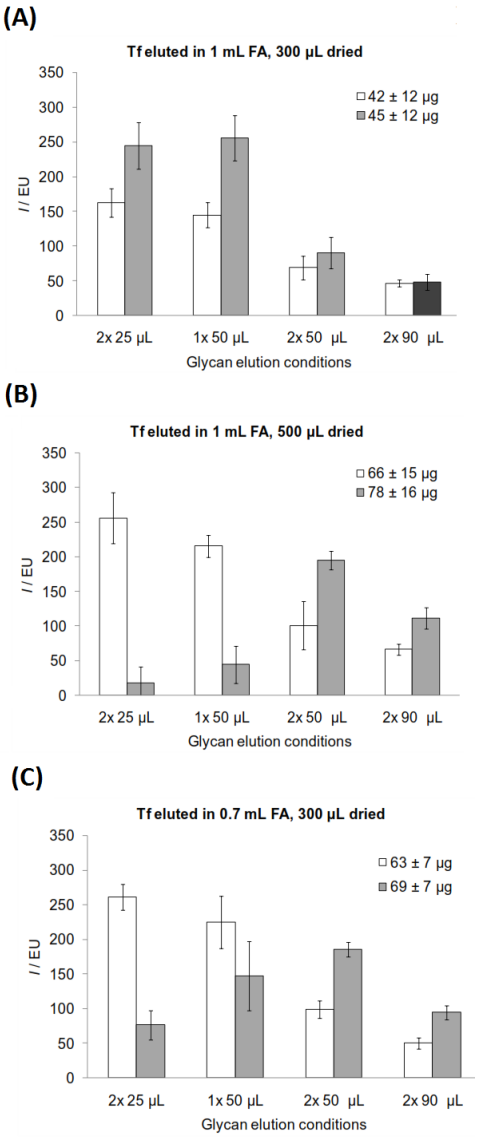

Figure 4. Optimization of transferrin isolation and glycan preparation conditions, according to Table 1. Previously used standard preparation conditions are shown in dark grey. White – dry Tf eluate samples were desalted with cold methanol before deglycosylation. Grey – dry Tf eluate samples were not desalted before deglycosylation. Average value and standard deviation of highest glycan peak intensity are shown, as well as the mass of protein used (n = 4). EU – emission unit.

IgG *N*-glycome is known to share some *N*-glycans with Tf *N*-glycome. Indeed, chromatograms of some the samples showed that small amount of IgG was co-purified with Tf. In addition, concentrated elution fraction samples analysed by SDS-PAGE contained additional bands when IgG was not removed from plasma sample before transferrin isolation (Figure 5). In order to eliminate the possible contamination and skewing of the results, we removed the IgG from initial plasma sample before the Tf isolation. Therefore, we suggest to use IgG depleted plasma for Tf isolation when UPLC is used for glycan quantification. Improved Tf purification (without visible IgG bands after fractions analysis by SDS-PAGE) was also achieved if 1 × PBS, pH 7.4 with increased NaCl

Table 2. Coefficients of variation (CV) of glycan chromatographic peak percent areas in relation to experimental conditions from Table 1 tested on larger number of samples (n = 24).

Peak percent area CV / %	Number of peaks		
	A	B	C
0–5	4	4	12
5–10	13	13	13
10–15	15	4	4
> 15	3	14	6

Experimental conditions (see Table 1): A – $V_{elution}$ = 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0, V_{dried} = 300 µL, $V_{glycan\ eluate}$ = 2× 25 µL of ultra-pure water. B – $V_{elution}$ = 1 mL of 0.1 mol L^{-1} formic acid, pH 3.0, V_{dried} = 500 µL, $V_{glycan\ eluate}$ = 2× 50 µL of ultra-pure water. C – $V_{elution}$ = 0.7 mL of 0.1 mol L^{-1} formic acid, pH 3.0, V_{dried} = 300 µL, $V_{glycan\ eluate}$ = 2× 50 µL of ultra-pure water.

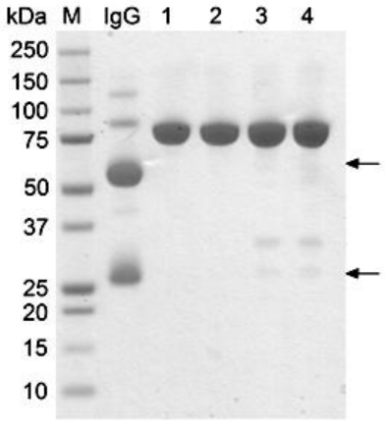

Figure 5. SDS-PAGE of 5× concentrated elution fractions after transferrin isolation from IgG depleted plasma (lanes 1 and 2) and plasma without IgG depletion (lanes 3 and 4). M – Precision Plus Protein™ Standards All Blue molecular mass standard (Bio-Rad). Arrows represent bands from contaminants in case when IgG was not removed from blood plasma before Tf isolation.

concentration (250 mmol L^{-1} instead of 137 mmol L^{-1}) was used to wash the unbound proteins before Tf elution (results not shown).

CONCLUSION

Here we describe for the first time a preparation and application of CIMac-@Tf 96-well monolithic plate for immunoaffinity purification of Tf. First, a successful technological transfer of the @Tf mAb immobilization from a single monolithic column to the plate format was accomplished. Next, the purification conditions for Tf isolation from human plasma were optimized together with the subsequent *N*-glycan preparation method., Thus, we obtained a clean sample that allowed highly sensitive HILIC-UPLC quantification of Tf *N*-glycans. The established method enables high-throughput Tf isolation. In the future, we plan to isolate Tf from up to one thousand blood plasma samples and analyze Tf *N*-glycans to assess the natural biological variability of Tf *N*-glycome of healthy individuals as well as to detect disease caused changes of the Tf *N*-glycome.

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