

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(PHD)**

**Identification of myeloma multiplex glyco-biomarkers by capillary
electrophoresis**

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Identification of myeloma multiplex glyco-biomarkers by capillary electrophoresis

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The Examination takes place at the Library of the Department of Physiology, Faculty of Medicine, University of Debrecen, 12:00 am, 04th May, 2018.

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I. INTRODUCTION

It is known that carbohydrates play an important role in various signaling and metabolic processes, as well as in proper folding of proteins, stability, quality control, cell wall formation, molecular recognition and immunity. In recent years, the significance of post-translational modifications has been recognized and the need to analyze complex carbohydrates has increased. O- and N-glycans are equal important in living organisms, however, the N-glycans are much more widely studied, as the specific enzymes required for the release of most O-glycans are not available. Knock-out mice were shown that the elimination of the carbohydrate moiety of glycoproteins may have serious health consequences. The lack or mutation of the glycosyl-transferases and glycosydases is lethal during embryonic development. The carbohydrate content of glycoconjugates varies, as the number of glycosylation sites and the occupancy at particular sites can vary within a protein (macroheterogeneity). In antibodies it is relatively low (2-5% of total mass), while other glycoproteins can be highly glycosylated such as haptoglobin (19%), erythropoietin (40%), α 1-acid glycoprotein (45%) and mucins (40-80%). Glycoproteins can have multiple glycosylation sites with different glycoforms on each site resulting in broad site-specific structural diversity (microheterogeneity) of protein glycosylation. The structural heterogeneity of glycans depends on the activity of glycosidases and glycosyl-transferases, which could be changed in pathological conditions. Glycosylation analysis offers a new avenue in biomarker discovery as glycosylation changes could be sensitive indicators of the actual biochemical state of an organism. With the help of glyco-biomarkers, diseases might be diagnosed at early stages and avoid time-consuming and invasive diagnostic procedures. Multiple myeloma (MM) is an inmedicable hematological disease of the human plasma cells, producing abnormal antibodies (referred to as paraproteins) leading to serious health issues. Due to abnormal bone marrow proliferation, normal bone marrow function gets damaged and severe anemia develops. The malignant bone marrow cells produce a huge amount paraprotein, therefore, causing serious acute or chronical kidney destruction. The paraproteins are abnormal immunoglobulin fragments or immunoglobulin light chains (kappa or lambda), which circulate in the blood system as abnormal stem cell proliferation clones. Malignant transformation proceeding during MM may alter the glycosylation of proteins, which enhances the expression of pro-inflammatory glycoforms. Analysis of the N-glycosylation profile of paraproteins may be prognostic in the treatment of myeloma multiplex.

There is a growing demand in the biopharmaceutical industry for large scale N-glycosylation analysis of biotherapeutics, especially monoclonal antibodies. Modern capillary electrophoresis devices are easy to use, but to further integrate them into the clinic, it is advisable to perform some further enhancements. Since the N-glycan sample preparation process can be automated, it would be possible to simultaneously and quickly analyze large quantities of samples by CE-LIF. The reductive amination derivatization method is widespread, which uses sodium cyanoborohydride to reduce the sugar-fluorophore Schiff-base. This labeling process is extremely efficient and easy to carry out, but hydrogen cyanide produced during the reaction representing a major source of concern in large scale applications. Previous studies have shown using both $[\text{RuCl}_2(\text{mtppps})_2]$ and $[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$ with excellent catalytic activity in the dehydrogenation processes and in the transfer hydrogenation of unsaturated substrates (aldehydes) and formic acid dissociation.

Williams et al. developed a sequential injection applied before analysis (SIBA) that significantly reduced analysis times without loss of resolution and increased the throughput for oligonucleotide analysis in capillary electrophoresis. Using the SIBA protocol, oligonucleotide components were electrokinetically injected before electrophoretic separation. Several alternate sample injection and separation solutions were published, but these methods were only suitable for separating one sample component.

II. AIMS AND MOTIVATION

Multiple myeloma patients produce more than 50% of IgG-derived paraproteins, the remainder being distributed in nearly identical proportions between the IgA and IgD subclasses. With this in mind, my aim was to investigate the glycosylation of total serum and serum IgG fraction in the capillary electrophoresis system by laser-induced fluorescence detection (CE-LIF) in patients with multiple myeloma and determine whether the method could help to establish accurate diagnosis of patients in clinical diagnostics.

I investigated the usefulness of $[\text{RuCl}_2(\text{mtppps})_2]$ and $[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$ complexes for the hydrogenation of the imine intermediates (reduction step) in the reductive amination process of glycan labeling. Human IgG, fetuin and ribonuclease B N-glycans (representing the three main N-glycan types of neutral, sialylated and high mannose) were suitable standards for the studies.

Recently, CE-LIF has become a robust and efficient separation platform requiring low sample quantities (fmol). My aim was to develop a new type of multiplex injection method, in which detailed information from the analysis could be retained with shorter separation time.

III. MATERIALS AND METHODS

III.1. Ethical License

Serum samples were collected at the Clinical Centre of Internal Medicine, University of Debrecen (Hungary) and the 2nd Department of Internal Medicine, Semmelweis University (Budapest, Hungary) with all necessary Ethical Committee approvals (HBR/052/00437-2/2015) from untreated, treated and remission multiple myeloma patients as well as healthy controls.

III.2. Human serum and standard glycoprotein samples

Included in the study, 6 untreated, 6 treated and 6 remission stage multiple myeloma patients as well as 6 male and 6 female healthy controls using clot activator containing serum tubes (BD, Franklin Lakes, NJ). MM patients who had acute infectious diseases, acute or chronic inflammatory diseases, other malignant cancer history besides MM, and drug abuse were all excluded from the study. The collected blood samples were centrifuged at 7500 x g for 30 min and the serum fractions were stored at -70 °C until further processing. Fetuin (fetal calf serum), ribonuclease B (bovine pancreas) and human serum immunoglobulin G were from Sigma-Aldrich (St. Louis, MO).

III.3. Chemicals and reagents

HPLC water, ethylenediaminetetraacetic acid (EDTA), sodium hydrogen carbonate, sodium hydroxide, glycine-HCl, sodium formate, acetic acid, cysteine, iodoacetamide and sodium cyanoborohydride (1 M in THF) were from Sigma-Aldrich (St. Louis, MO). G7 buffer and NP-40 solution were from New England Biolabs (Ipswich, MA). PNGase F was from ProZyme (Hayward, CA) and papain from AppliChem (Darmstadt, Germany). The 8-aminopyrene-1,3,6-trisulfonate (APTS), the maltooligosaccharide ladder and the NCHO Carbohydrate Labeling and Analysis kit were from SCIEX (Brea, CA). The NEB Denaturation Buffer, the 1x-Glyco Buffer 2 and the 1% NP-40 solution were from New England Biolabs (Ipswich, MA). The Agencourt CleanSEQ magnetic beads were from Beckman Coulter (Indianapolis, IN).

III.4. Methods

For the analysis of multiple myeloma samples, I developed a method for analyzing serum IgG N-glycosylation and another one for N-glycan analysis from total serum. Patients involved in myeloma multiplex were classified into three main groups:

- 1) *untreated patients* - all newly diagnosed patients who have not yet been treated and have been classified as ISS II on the basis of their clinical examinations;
- 2) *treated patients* - received selective therapy for the treatment of multiple myeloma at the time of sampling;
- 3) *remission patients* - at the time of sampling they were in the asymptomatic phase of their illness.

The members of the control group were healthy middle aged people, with matched age and gender.

During processing of the standard glycoproteins, I have used the methods already published by our research group.

III.4.1. Papain digestion

One ml papain solution (10 mg/ml papain in 1xPBS Sigma-Aldrich, St. Louis, MO) was mixed with 4 ml of 0.2 M EDTA and 0.2 M cysteine containing 1 x PBS buffer (pH 6.5). 100 μ L of this reagent was added to 100 μ L of serum sample and incubated at 37 °C for 4 h. The reaction was stopped by the addition of 20 μ L iodoacetamide solution (0.3 M in 1 x PBS).

III.4.2. Partitioning of the Fc fragment

220 μ L of the papain digest was used for all sample types (controls, untreated, treated and remission stage multiple myeloma patients) after purification on 20 μ L bed-volume Protein A affinity microcolumns (PhyNexus, San Jose, CA). 200 μ L of 1 x capture buffer (0.7 M NaH_2PO_4 , 0.7 M NaCl, pH 7.4) was added to the serum samples and then the resin was washed with 200 μ L of 0.7 M NaH_2PO_4 0.7 M NaCl buffer (pH 7.4). Then 200 μ L of wash buffer (140 mM NaCl) was added to the microcolumns and the Fc fragments were eluted with 200 μ L of 15% acetic acid. The samples were transferred to 10 kDa spin-filters (VWR, Radnor, PA) and centrifuged at 11270 x g for 10 minutes. Then 100 μ L of HPLC grade water was added and again centrifuged at 11270 x g for 10 minutes to remove any remaining acetic

acid. The N-glycan moiety of the partitioned Fc fractions was PNGase F digested in situ on the filter and the liberated N-glycans were APTS labeled as described below.

III.4.3. κ/λ light chain partitioning

220 μL of the digestion mixture (as described above) was used for all samples (controls as well as untreated, treated and remission stage multiple myeloma patients) and purified on CaptureSelect LC-kappa (Hu) and CaptureSelect LC-lambda (Hu) filled affinity microcolumns (20 μL bedvolume, PhyNexus). 200 μL of capture buffer (1 x PBS) was added to the serum samples and then the resin was washed with 200 μL of 1 x wash buffer I (0.7 M NaH_2PO_4 0.7 M NaCl pH 7.4). Then 200 μL of 1 x wash buffer II (140 mM NaCl) was added to the microcolumns and the IgG kappa/lambda light chains were eluted with 200 μL of 0.1 M glycine-HCl (pH 2.0). The samples were then transferred to 10 kDa centrifugal filters, centrifuged at 11270 x g for 10 minutes followed by the addition of 100 μL of HPLC grade water and centrifugation again 11270 x g for 10 minutes. The prepared light chain fractions were PNGase F treated in situ on the filter and APTS labeled as described below.

III.4.4. N-glycan digest of glycoproteins and fluorophore labeling of the released sugars

1 μL of serum from each patient (healthy controls as well as untreated, treated and remission stage multiple myeloma patients) was diluted by 10 μL of HPLC grade water (Millipore, Darmstadt, Germany) then 1 μL of denaturing buffer (400 mM DTT, 5% SDS) was added followed by incubation at 65 $^\circ\text{C}$ for 10 minutes. The reaction mixture was filtered through 10 kDa spin-filters (VWR, Radnor, PA) at 11270 x g for 10 minutes and washed by 100 μL of HPLC grade water. Both the denatured serum samples as well as the Fc and κ/λ light chain fractions were digested *in situ* on the filters by the addition of 29 μL of NaHCO_3 buffer (20 mM, pH 7.0) and 1 μL of PNGase F (200 mU, 2.5 U/ml, Prozyme), followed by incubation at 37 $^\circ\text{C}$ overnight. The released N-glycans were centrifuged through 10 kDa spinfilters at 7500 x g for 10 minutes and dried in SpeedVac (Thermo Scientific, Schaumburg, IL) prior to the fluorophore labeling step.

6 μL of 20 mM 8-aminopyrene-1,3,6-trisulfonic acid in 15% acetic acid and 2 μL of NaCNBH_3 (1 M in THF) were added to the dry pellet and incubated at 37 $^\circ\text{C}$ overnight. The labeled samples were purified using the Fast Glycan Sample Preparation and Analysis kit (SCIEX, Brea, CA) following the protocol and immediately used for CE-LIF analysis or stored at -20 $^\circ\text{C}$ for later analysis.

III.4.5. Standard glycoprotein sample preparation

Fetuin, RNase B and IgG samples (10 mg/ml) were first denatured with 1 μ L denaturation buffer (400 mM DTT, 5% SDS), followed by PNGase F digestion at 60 °C for 10 min. Then 2.5 μ L G7 buffer, 2.5 μ L NP-40 solution and 1 μ L PNGase F enzyme were added to the samples and digested at 60 °C for 20 minutes. The released glycans were fluorescently labeled with APTS and our earlier described magnetic bead based purification protocol was applied both after glycan release and fluorophore labeling.

III.4.6. $[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$ (trisodium cis-mer-chloro-dihydrido-tris(diphenylphosphinobenzene-m-sulfonato) iridium(III)) and $[\text{RuCl}_2(\text{mtppps})_2]$ dichloro-bis(diphenylphosphinobenzene-m-sulfonato)ruthenium(II) compounds

$[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$ (trisodiumcis-mer-chloro-dihydrido-tris (diphenylphosphinobenzene-m-sulfonato)iridium(III)) and $[\text{RuCl}_2(\text{mtppps})_2]$ (disodium dichloro-bis (diphenylphosphinobenzene-m-sulfonato)ruthenium(II)) were made in house (Department of Physical Chemistry, University of Debrecen, Hungary). Please note that the Ru(II)-complex has a dimeric structure in solid state. Both complexes were highly water-soluble and could be synthesized easily from their halide precursors of $\text{IrCl}_3 \cdot 3\text{H}_2\text{O}$ and $\text{RuCl}_3 \cdot 3\text{H}_2\text{O}$, respectively, in reaction with monosulfonated triphenylphosphine (mtppps).

III.4.7. APTS labeling with iridium and ruthenium complexes

The following reaction mixture was used as derivatization control: 6 μ L of 40 mM 8-aminopyrene-1,3,6-trisulfonic acid in 20% acetic acid and 1.5 μ L of 1 M sodium cyanoborohydride (in THF) were added to the dried sugars and incubated at 55 °C for 60 min. For Ir- or Ru-complex based transfer hydrogenation mediated reductive amination, 6 μ L of 40 mM 8-aminopyrene-1,3,6-trisulfonic acid in 20% acetic acid and 1.5 μ L of concentrated (98%) formic acid, 1.5 μ L of water-soluble iridium(III)dihydride complex (5 mg/mL) or 1.5 μ L of water-soluble ruthenium complex (5 mg/mL) were added to the dried sugar samples followed by incubation at 55 °C, as specified under the actual application descriptions. The labeled samples were partitioned from the excess labeling dye with the magnetic beads provided in the Fast Glycan Sample Preparation and Analysis kit (SCIEX, Brea, CA) and immediately used for CE-LIF analysis or stored at -20 °C.

III.4.8. Capillary electrophoresis

A P/ACE MDQ System (SCIEX, Brea, CA) was used to perform all capillary electrophoresis analyses. The separations were monitored by laser induced fluorescence (LIF) detection using a 488 nm Ar-ion laser with a 520 nm emission filter. 20 cm or 50 cm effective length (30 cm or 60 cm total) 50 μm i.d. (365 μm outer diameter) NCHO capillaries were employed with the NCHO separation gel buffer system (both from SCIEX, Brea, CA) for the separations. 50 cm effective length (60 cm total) 50 μm i.d. BFS capillaries were employed with the NCHO separation gel buffer system (both from SCIEX) for alternative labeling analysis. Samples were pressure injected by 1 psi (6894 Pa) for 5 seconds. The separations were accomplished in reversed polarity mode by applying 30 kV electric field strength. The 32 Karat software (SCIEX, Brea, CA) was used for data acquisition and processing. The applied separation voltage was 30 kV in reversed polarity mode (anode at the detection side) at 25 °C or 37 °C cartridge temperature, depending on the experiment in the separation window dependent multiple injection method. For injection either a one-step pressure injection was used: 1.2 psi for 0.15 min; or a three-step electrokinetic injection: water: 0.1 psi for 0.1 min followed by the sample: 2.3 kV for 0.1 min and a buffer push: 0.1 psi for 0.1 min.

III.4.9. Statistical analysis

Principal component analysis (PCA) and one-way analysis of variance (ANOVA) were performed with SPSS 22 (IBM Corp.) using PeakAreas% as input derived from 32 Karat software (SCIEX, Brea, CA). Tukey *post hoc* test was used to compare peak intensities between the experimental groups. Differences between means at $P < 0.05$ were considered as significant.

IV. RESULTS

IV.1.1. Comparison of the serum N-glycome profile between the healthy control and MM groups

First the global serum N-glycan profiles of the healthy control samples were analyzed. 14 peaks of interest were identified (peak area $\geq 1\%$) including multisialo (1-4), monosialo (5-8) and neutral glycans (9-14).

After specifying the main peaks of interest in the healthy group, the global serum N-glycan profiles of the three patient groups of untreated freshly diagnosed, treated and remission stage patients were analyzed and the results were compared. I found that the groups showed major differences in peak distribution. Freshly diagnosed untreated patient group showed significant changes in the sialoform (peaks 1-8) to neutral (peaks 9-14) carbohydrate ratio, referred to as sialoform/neutral-form. The sialoform/neutral value in the control total serum glycome level was 2.97 (74.78%/25.22%), while in the untreated patient sample it was only 0.39 (28.16%/71.84%), thus practically the opposite as of in the control serum, demonstrating a striking change.

IV.1.2. N-glycosylation profiling of the Fc and Fab κ/λ light chain fractions

Next I narrowed my focus to study the N-glycosylation of the Fc and Fab κ/λ light chain fractions that were partitioned by Protein A and κ/λ light chain specific microcolumn affinity pulldowns after papain digestion.

It is well known that in the serum N-glycome, most neutral species are originated from the approximately 25% immunoglobulin fraction. Please note that the total amount of immunoglobulins in ISS stage II patients was in the range of 5-7 g/dL compared to the normal range of 0.7-1.6 g/dL with the associated albumin level in both around 3-5 g/dL. Interestingly, the changes in the N-linked glycan profile of the immunoglobulin Fc fractions were not as substantial as was observed in the global serum N-glycan profiles, except the peak ratio changes in comparison to the sialoforms (peaks 1-8), in which case all three patient sample traces showed significantly increased neutral glycan species (peaks 9-14). The sialoform/neutral form ratios were as follows: control IgG sialoform/neutral form= 0.22 (18.26%/81.74%), while in the untreated patient samples sialoform/neutral form= 0.08 (7.49%/92.51%); treated patients sialoform/neutral form= 0.11 (10.19%/89.81%) and in the remission stage sialoform/neutral form= 0.14 (12.55%/87.45).

I analyzed the released and APTS labeled N-linked glycan profiles from the Fab kappa (κ) and lambda (λ) light chains. Here no sialoforms were detected in the control group sample, i.e., the neutral form was 100%. The freshly diagnosed untreated patient group sample showed a few sialylated glycans (peaks 6, 15 and 16): sialoform/neutral form= 6.96 (87.44%/12.56%). The N-glycan profile of the treated and remission stage groups showed no sialylated structures on the lambda and kappa fragments, i.e., the neutral form represented 100% in both instances.

IV.1.3. Statistical analysis

During the statistical analysis, first the total serum N-glycosylation data was evaluated. In this case the first two principal component axes accounted for 47.3% and 17.2% data variance respectively, representing 64.5% of data variance cumulatively, which was sufficient to classify the data into four distinct groups of control, untreated, treated and remission.

In case of the papain digested IgG fragments, the first two principal component axes accounted for 45.6% and 16.1% data variance respectively, representing 61.7% of data variance cumulatively, which was only sufficient to distinguish the control group from the MM patients, but not within the latter ones.

One-way analysis of variance (ANOVA) revealed statistically significant differences between the studied groups in mean PeakArea% as described inhere:

- in the total serum samples: A3G(4)3S(6,6,6)3; A2G(4)2S(6,6)2; A2G(4)2S(3,3)2; F(6)A2BG(4)2S(6,6)2; A2[6]G(4)2S(6)1; F(6)A2G(4)2S(3)1; F(6)A2GB(4)2S(3)1; F(6)A2B; F(6)A2[6]G(4)1; F(6)A[3]G(4)1; F(6)A2[6]BG(4)1; F(6)A2G(4)2.
- and in the IgG Fc fragments: F(6)A2G(4)2S(3)1; F(6)A; F(6)A2B; F(6)A[3]G(4)1; F(6)A2[6]BG(4)1; F(6)A2BG(4)2.

I had to consider the sampling distribution to determine how accurate the conclusions were based on the N-glycan profiles. When the average population showed normal distribution, the average sampling distribution was followed by normal distribution, where the mean of the sample equaled the population actual parameter. The standard error is the standard deviation of the sampling distribution as outlined above, so it shows that when we take a random sample of several identical elements from a population, the average of the samples is spread around the population parameter. The smaller the standard error value, the closer we approximated the real average value. Based on my calculations, the standard error value was

insignificant (less than 1%), so my results apparently represented the actual value of the average.

IV.2.1. Fluorophore labeling efficacy of iridium and ruthenium phosphine complexes

Transfer hydrogenation ($\text{HCOOH} \rightarrow \text{H}_2 + \text{CO}_2$) catalyzed by $[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$ and $[\text{RuCl}_2(\text{mtppps})_2]$ were evaluated as alternatives to reduction by NaBH_3CN during fluorophore labeling of simple and complex carbohydrates by reductive amination. A standard maltooligosaccharide ladder and endoglycosidase released N-glycans from fetuin, ribonuclease B and human serum were labeled using the aforementioned two catalysts mediated transfer hydrogenation approach. The resulting APTS labeled samples were analyzed by CE-LIF. The electropherograms of the standard maltooligosaccharide ladder using different reducing agent mediated labeling were compared. For quantitative evaluation, the average peak heights of the maltopentaose (G5 oligomer) were determined. The intensities for APTS labeled maltopentaose peaks were very comparable for $[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$ and NaBH_3CN , while the use of $[\text{RuCl}_2(\text{mtppps})_2]$ in the same molar concentration resulted in lower intensities.

IV.2.2. Fluorophore labeling of biological samples with iridium and ruthenium complexes

To test transfer hydrogenation as an alternative and efficient reduction procedure on biologically relevant samples, N-glycans enzymatically released from fetuin, ribonuclease B and human serum were labeled using NaBH_3CN , as well as transfer hydrogenation from formic acid catalyzed by $[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$ and $[\text{RuCl}_2(\text{mtppps})_2]$. CE-LIF electropherograms showed similar labeling efficiencies for NaBH_3CN and $[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$. $[\text{RuCl}_2(\text{mtppps})_2]$ could be also used effectively, however, for similar labeling performance it had to be applied in higher (2.5 fold) concentration. It is important to note that in case of the analysis of the fetuin derived highly sialylated N-glycans, the peak at 11 min was larger, suggesting better stability against sialic acid loss. For the N-glycan profiles of ribonuclease B and human serum glycoproteins, all of the three types of the aforementioned labeling procedure achieved the same labeling efficiency.

IV.3.1. Principle of separation window dependent multiple injection (SWDMI)

Sequential injections and large-scale methods are required in the biotechnology industry for clone selection analysis, for example in the analysis of IgG N-glycans. Separation Window Dependent Multiple Injection (SWDMI) utilized a serial injection concept during the electrophoresis process by periodically interrupting the separation to introduce consecutive samples with expectedly similar peak profile, e.g., IgG N-glycans during clone selection. Thus, the number of actual separation steps was equal to the number of samples injected in SWDMI. Separation window is the complete migration time segment needed to separate all sample components after sample injection. Injection of each succeeding sample was implemented when the first peak of the previously injected sample was detected, considering the predetermined separation window. In SWDMI both pressure and electrokinetic injection methods were available. During the experiments, commercially available standard IgG was used since the conserved glycosylation pattern of this glycoprotein was well characterized and regularly used for method validation and testing.

IV.3.2. SWDMI by pressure injection

The CE-LIF profile of four IgG N-glycans was used to adjust the pressure-injected SWDMI separation method. The analysis was performed on a 20 cm effective length capillary. The separation was interrupted for each sample injection when the first peak of the mixture was detected, in this case the APTS peak at 1.28 min. The separation was started by applying 30 kV (0.1 min ramp time) for 0.15 min at 1.2 psi after the injection of the first sample. Once the unreacted APTS peak was detected, the separation process was stopped and the second sample was injected by the same pressure parameters followed by immediate application of the separation voltage. The peaks corresponding to the IgG glycan structures of the first sample started to appear at 2.61 min in the first cycle regime and the separation window was 1.14 min long. Please note that the actual separation of the sample components was not interrupted by the consecutive injection processes while they passed the detection window, therefore the peak pattern was always adequately visualized. In this instance, due to the precise determination of the separation time window (1.14 min), the APTS peak of the second sample migrated rather close to the last peak of the first IgG sample, at 3.77 min. The second sample was injected in the middle of the first cycle and the first cycle ended in the middle of the separation of the second cycle sample. Then I injected the third sample according to the injection parameters of the second sample and, accordingly, the fourth sample was injected

when the APTS peak of the second sample was detected during the third cycle. To follow this concept, full analysis of four samples took in less than 10 min. Please note the four complete separation cycles would have taken for 36 minutes for the same four samples.

IV.3.3. SWDMI by electrokinetic injection

Electrokinetic injection with the SWDMI process was required to analyze highly diluted samples or when high viscosity gel-buffer systems were used for the separation. This approach resulted in good signal to noise ratio, even from low concentration samples. In addition to IgG carbohydrates, free APTS was seen, which was due to the electrokinetic injection. As part of the electrokinetic injection process, a depleted ion zone (water injection) was electrokinetically introduced prior to the sample injection to support transient isotachopheresis based sample pre-concentration for dilute analytes. A pressure mediated buffer push was also applied after each sample injection to properly terminate the injection zones. With the results of using the electrokinetic injection method of SWDMI, I would like to emphasize that, in the case of electrokinetic injection, the tray movements associated with the two extra injection steps (HPLC water pre-injection, and post-injection buffer push) required a longer time than pressure sample injection. For this reason the separation of 96 samples for 288 minutes using electrokinetic injection, while the same sample analysis only took 240 minutes using the pressure-based SWDMI method. Therefore, when sample concentration is not an issue and/or low viscosity separation matrix is used, pressure mediated SWDMI method is more advisable to achieve faster analysis.

IV.3.4. Effect of the separation temperature in SWDMI

All of the above described multiple injection and separation methods were executed at 25 °C capillary cartridge temperature. Further reduction of the overall analysis time with multiple injections was attempted by increasing the separation temperature to 37 °C. The N-glycan peaks (FA2, FA2(6)G1, FA2(3)G1, FA2G2) considered to be important for analysis were well separated from each other at this higher temperature. It is important to note that the peak ratios of the IgG N-glycan profiles remained unchanged. The overall separation time at the elevated temperature was approximately 230 min for 96 samples (including all injection and separation related tray movements) with the peak area reproducibility of 10.75% RSD. The reproducibility of the migration time was below 0.5% RSD.

V. CONCLUSIONS

V.1. Serum N-glycosylation of patients with multiple myeloma

In my studies, I compared the serum N-glycan samples of healthy control serum samples with patients of multiple myeloma (freshly diagnosed untreated, treated and remission). The investigation included the N-glycosylation analysis of human serum proteins at the global level as well as the Fc and Fab κ/λ chain fractions of papain digested paraproteins. Principal component analysis clearly separated the four study groups (control, freshly diagnosed untreated, treated and remission MM patients) based on the results of the total serum N-glycosylation analysis. However, the N-glycosylation profile of the papain-digested IgG lambda and kappa fragments did not show such significant and obvious differences. Here only differences between healthy controls and those with multiple myeloma were clearly seen.

The ratio of sialylated and neutral glycoforms was significantly changed on serum N-glycome levels. One-way analysis of variance (ANOVA) showed statistically significant changes ($p < 0.05$) for 12 serum N-glycans, while 6 N-glycans presented significant differences at the IgG fragment level.

Based on my results, I found that the total serum N-glycosylation profile offered higher information level and the procedure was simpler and faster because it did not require extra digestion and purification steps, so it could be easier applied in a clinical setting.

V.2. Alternative reducing agents

During the capillary electrophoresis of carbohydrates, almost exclusively charged fluorophore labeling agents have been applied via reductive amination reaction. I studied water-soluble Ir (III) and Ru (II) phosphine complexes, namely formic acid hydrogenation processes catalyzed by $[\text{IrH}_2\text{Cl}(\text{mtpms})_3]$ and $[\text{RuCl}_2(\text{mtpms})_2]$ most commonly, as an alternative to the NaBH_3CN reductive amination labeling process. The use of $[\text{IrH}_2\text{Cl}(\text{mtpms})_3]$ during the reductive amination reaction showed very similar tagging efficiencies as with the use of conventional NaBH_3CN . Conversely, the cheaper $[\text{RuCl}_2(\text{mtpms})_2]$ showed slightly lower activity compared to NaBH_3CN , but at higher concentrations (2.5 x), I achieved the desired yield. Notably, this procedure did not release acutely toxic hydrogen cyanide, therefore, it was more environmentally friendly and eliminated the health risks, which accompanied the use of NaBH_3CN as reducing agent in large scale use of reductive amination reactions.

V.3. Separation window dependent multiple injection

New generation protein therapeutics represent a growing current trend in the biopharmaceutical industry. More than half of these biotherapeutics are monoclonal antibodies and the analysis of their sugar moieties requires rapid and high-throughput separation methods. During my work, I developed a separation window-dependent multiple injection method that could be applied for both in pressure and electrokinetic injection methods. The efficacy of the method was demonstrated by capillary electrophoretic analysis of the main N-glycan structures of IgG. I defined the separation window as the migration time, which included the actual separation of all sample components (did not include injection time), including a peak representing the free APTS dye in the sample, which was the starting point for the separation time window. I performed consecutive injections when the APTS peak of the previously injected sample reached the detector window. The efficiency of the approach was verified using a single channel CE-LIF system by analyzing 96 samples. The method was easy to apply and significantly shortened the analysis of 96 samples from 864 minutes to 288 minutes. Please note that in electrokinetic injection mode the procedure required longer time (288 minutes for 96 samples) due to some extra tray movements of device. In pressure injection mode, it had less tray movement, hence the entire process time was shorter (240 minutes for 96 samples) approaching the actual time needed to separate the samples. In this case only minimal tray movements were required in the CE-LIF apparatus, which took minimal time to implement, thus, the sample separation was started immediately. The total process time was further reduced by increasing the separation temperature from 25 ° C to 37 ° C, reducing the total analysis time to 230 minutes for 96 samples.

VI. SUMMARY

During my work, I studied the N-glycosylation of Fc and Fab κ / λ fragments of total serum and IgG of myeloma multiplex patients by CE-LIF to determine which test method would be better suited to provide results of practical clinical diagnostics interest. The ratio of sialylated and neutral N-glycoforms in total serum and IgG paraprotein Fc and Fab κ / λ fragments was significantly altered at the N-glycan level. Statistical data processing showed that the total serum N-glycosylation possessed higher information content since all patient groups (newly diagnosed, untreated, treated and remission) were clearly separable. This information was of

great significance because allowed accurate ISS classification of patients, i.e., selection of a proper treatment strategy, thus achieving longer survival and better quality of life.

I also developed an alternative fluorophore labeling method for the pharmaceutical industry to examine glycoforms by using transfer hydrogenation with $[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$ and $[\text{RuCl}_2(\text{mtppps})_2]$ in the reduction amination reaction of carbohydrates by APTS. The $[\text{IrH}_2\text{Cl}(\text{mtppps})_3]$ exhibited nearly the same labeling efficiency as of the conventional NaBH_3CN , while the much cheaper $[\text{RuCl}_2(\text{mtppps})_2]$ provided the desired yield, only in slightly higher concentrations (2.5 x). The use of these agents did not cause the release of acutely toxic hydrogen cyanide, therefore, offered more environmentally friendly solutions and eliminated the health risks, which accompanied the use of NaBH_3CN in large scale applications.

Finally, I developed a separation window dependent multiple injection (SWDMI) method for easier and faster CE-LIF analysis of a large number of samples. The separation window was determined as the migration time window covering the actual separation of all sample components including the peak representing the APTS dye, what was the starting point of the time window. Every consecutive injection was implemented at the time point when the APTS peak of the preceding injection reached the detection point. The SWDMI method required longer processing time when electrokinetic injection was used (288 minutes for 96 samples). By the application of pressure injection, the total processing time was shorter due less tray movement requiring, only 240 minutes for 96 samples. Increasing the separation temperature from 25 to 37 °C, further reduced the total processing time to 230 min.



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List of publications related to the dissertation

1. **Kovács, Z.**, Papp, G., Horváth, H. H., Joó, F., Guttman, A.: A novel carbohydrate labeling method utilizing transfer hydrogenation-mediated reductive amination.
142, 324-327, 2017.
DOI: <http://dx.doi.org/10.1016/j.jpba.2017.05.017>
IF: 3.255 (2016)
2. **Kovács, Z.**, Simon, Á., Szabó, Z., Nagy, Z., Váróczy, L., Pál, I., Csánky, E., Guttman, A.: Capillary electrophoresis analysis of N-glycosylation changes of serum paraproteins in multiple myeloma.
Electrophoresis. *38* (17), 2115-2123, 2017.
IF: 2.744 (2016)
3. **Kovács, Z.**, Szarka, M., Szigeti, M., Guttman, A.: Separation window dependent multiple injection (SWDMI) for large scale analysis of therapeutic antibody N-glycans.
J. Pharm. Biomed. Anal. *128*, 367-370, 2016.
DOI: <http://dx.doi.org/10.1016/j.jpba.2016.06.002>
IF: 3.255





List of other publications

4. **Kovács, Z.**, Szarka, M., Kovács, S., Boczonádi, I., Emri, T., Abe, K., Pócsi, I., Pusztahelyi, T.:
Effect of cell wall integrity stress and RlmA transcription factor on asexual development and autolysis in *Aspergillus nidulans*.
Fungal Genet. Biol. 54, 1-14, 2013.
DOI: <http://dx.doi.org/10.1016/j.fgb.2013.02.004>
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