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

NEW ASPECTS IN ANALYSIS OF *N*-GLYCANS USING CAPILLARY GEL ELECTROPHORESIS

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The Examination takes place at the Library of the Department of Physiology, Faculty of Medicine, University of Debrecen at 12:00 am, 2nd May, 2017

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 14:00, 2nd May, 2017

1. INTRODUCTION

Electrophoresis covers a number of bioseparation tools where charged materials are separated due to differences in migration speed under the influence of an electrical field. This separation and chemical analysis technique was born in 1937 with the work of a Swedish biochemist called Arne Tiselius, who won the Nobel Prize for Chemistry in 1948. Since the 1950s, slab gel electrophoresis has been routinely used by analysts to separate and identify large biopolymers such as nucleic acids, proteins and complex carbohydrates. Although it is one of the most widely used separation techniques in molecular biology and biochemistry labs, slab gel electrophoresis generally suffers from long analysis times, low efficiencies and difficulties in detection and automation.

Capillary gel electrophoresis (CGE), on the other hand, is fast becoming the separation and characterization technique of choice in the bioanalytical field, which combines the advantages of liquid chromatography and 'conventional' slab/rod gel electrophoresis. In addition, this technique is not only used for the separation of biopolymers, but also in other areas, where has not earlier used the electrophoresis (e.g., food analysis, forensics and environmental protection, etc.). In addition, CGE is a reliable, fully-automated approach, offering rapid separations with high sensitivity, excellent resolution, ruggedness and ease of operation for the analysis of biologically important macromolecules, which contributed to the earlier completion of the Human Genome Project (HUGO) than originally planned. Although capillary electrophoresis (CE) first emerged as a free solution method, sieving media for size selective separations were developed soon after (such as sieving matrices made of agarose, polyacrylamide, polyethylene oxide, polyvinylpyrrolidone, sodium dodecyl sulfate, and so on). In case of CGE, the narrow-bore capillaries are filled with cross-linked gels or linear polymer sieving matrices for the separation. In addition, CGE technique can be used for the investigation of small molecule interactions and enzyme kinetic reactions.

At present, existing CGE methods can be readily transferred from the capillary format to electrophoresis microchips, i.e. lab-on-a chip, which offers rapid and effective separations for the biologically important macromolecules.

2. AIMS AND MOTIVATION

Capillary gel electrophoresis (CGE) is a rapidly developing technique, which is routinely used mostly in biopharmaceutical and biomedical analyses. This dissertation is started with a summary of the theoretical and practical aspects of CGE including separation principles, instrument design, capillary coatings, and the most frequently used gel-buffers. It is followed by the description of various detection options, such as UV-Vis, diode-array, laser-induced fluorescence and light-emitting diode-induced fluorescence types. Then, an overview of the key application areas is given with the main emphasis on nucleic acids, proteins and complex carbohydrate analysis, as well as the latest developments in microchip electrophoresis devices. The aims of this study were as follows:

1. To introduce a novel single-channel capillary gel electrophoresis system with microball ended emission optical fiber based LED-induced fluorescence detection design. To evaluate the suitability of this separation system, the degradation level of a large number (cc 1000) gDNA samples were analyzed to assess their quality, focusing on migration time reproducibility, limit of detection, as well as detector linearity in comparison to conventional agarose slab gel electrophoresis.

2. After the reproducibility studies, the aim was to develop a haplotyping (i.e. simultaneous multiple genotyping) method of two adjacent miRNA-binding SNPs (*rs1046322* and *rs9457*) in Wolfram syndrome-1 gene (WFS1) by combining double-tube allele-specific amplification and rapid capillary gel electrophoresis with LED-induced fluorescent detection to analyze the resulting DNA fragments.

3. The increasing use of glyco-markers and the huge market of therapeutic monoclonal antibodies requires the generation of synthetic carbohydrate antigens. In the third part of this study, the aim was to investigate polyclonal antibody response for newly synthesized maltose-BSA conjugates. First of all, a simple carbohydrate, maltose, was conjugated to a carrier protein (BSA) by reductive amination to improve their immunogenicity. After that, the synthetized neoglycoproteins were investigated by SDS-CGE as well as MALDI-TOF MS and then induced immune response against themselves. This elaborated method may be utilized in order to generate monoclonal antibody libraries for the discovery of mAbs in

normal and disease models with unique sugar specificities, which can be readily applied to biomedical research and clinical diagnostics.

4. In the fourth part of this study, the aim was to introduce an 8-aminonaphtalene-1,3,6-trisulfonic acid (ANTS) oligosaccharide database for capillary gel electrophoresis using LED-induced fluorescent detection. The validation of the generated GU values in the database was verified with *N*-glycans released from human immunoglobulin G and bovine pancreatic ribonuclease B. Currently, there are no structural databases for CGE-LEDIF separations, which contributed to the novelty and importance of this topic. In the absence of database, the migration properties must be calculated with the use of purified *N*-linked carbohydrate standards or in conjugation with other analytical techniques (e.g., NP-HPLC, MS, etc.). However, this generated database may prove useful for fellow separationists in the biomarker research and drug development. It is not necessary to separate large carbohydrate standards before each evaluation, thus, the unknown structures can be identified based on the migration times of the peaks of standards.

5. Finally, the activation energy concept related to the electromigration of carbohydrates were studied in CGE-LIF using linear (sugar oligomers with α1-4 linked glucose) and branched (sialylated, neutral and core fucosylated IgG) *N*-glycans. The aim of this study was to determine the electrophoretic mobility shift between the different structure oligosaccharides at different temperature/background electrolyte. The APTS-labeled oligosaccharides were separated in the temperature range of 20 – 50ºC, using either viscosity modifier (ethylene glycol) or polymeric additive (linear polyacrylamide, polyethylene oxide) containing BGEs. Previous reports have not noticed the effect of separation temperature on structure specific glycan migration using viscosity modifiers and/or polymeric additives containing BGEs. These factors may influence the stability of structural database, which contributed to the novelty and importance of this topic.

3. MATERIALS AND METHODS

3.1. Chemicals and Reagents

5-amino-2-napthalenesulfonic acid (ANSA), D-(+)-maltose monohydrate, human immunoglobulin G (hIgG), bovine ribonuclease B (RNase B), ethylene-glycol, polyethylene-oxide, dithiothreitol, iodoacetamide, and all other chemicals were also purchased from Sigma-Aldrich (St. Louis, MO, USA). 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and purified *N*-linked carbohydrate standards were from Prozyme (Hayward, CA, USA). The linear polyacrylamide solution was purchased from Polysciences (Warrington, PA, USA). High purity 8-aminopyrene-1,3,6-trisulfonate (APTS) and maltooligosaccharide ladder were obtained from SCIEX (Brea, CA, USA). All buffers and reagents were filtered through 0.45-μm-pore size Acrodisc syringe filters (Millipore, Billerica, MA, USA) and degassed prior to use.

3.2. Large-Scale Genomic DNA Analysis by CGE and slab gel electrophoresis

For large-scale genomic DNA (gDNA) analysis, the samples were from the Clinical Genomics Center (Debrecen). For CGE-LEDIF separation, DNA sizing ladder was used in the size range of 75 – 20 000 bases. For the migration time reproducibility study, DNA molecular mass marker was applied in the size range of 100 – 3 000 bases (Biocenter, Szeged, Hungary). CGE-LEDIF separations were accomplished by a novel single-channel DNA-CE instrument (BiOptic, New Taipei City, Taiwan) containing a 75 μm i.d. (360 μm o.d.) bare fused silica capillary with the effective separation length of 11 cm (total length: 15 cm). To accommodate fluorescent detection, the gel-buffer system contained ethidium-bromide. All separations were carried out at ambient temperature by applying 8 kV (533 V/cm) with a high-voltage power supply (EMCO, Sutter Creek, CA, USA). Data analysis was performed using the Q-Analyzer software package (BiOptic). DNA sizing marker was used in the size range of 75 – 20 000 bases for the agarose slab gel electrophoresis. All separations were performed in an E-Gel iBas Power System and the E-Gel Safe Imager Real-Time Transilluminator (Thermo Fisher Scientific, Darmstadt, Germany). Precast agarose gels contained 2% agarose with ethidium bromide. The separated DNA bands were visualized by a blue light transilluminator in real time. Data analysis was performed using the imaging system (Alpha DigiDoc AD-1201, Bio-Science, Budapest, Hungary) equipped with an Olympus digital camera with appropriate filters (C-4000ZOOM, Olympus, Budapest, Hungary).

3.3. Molecular Haplotype Analysis

3.3.1. Allele-Specific PCR

The gDNA samples were from the Department of Medical Chemistry, Molecular Biology and Pathobiochemistry (Budapest, Hungary). The study protocol was approved by the Local Ethical Committee (ETT-TUKEB ad.328/KO/2005, ad.323-86/2005-1018EKU from the Scientific and Research Ethics Committee of the Medical Research Council). gDNAs were acquired using non-invasive DNA sampling with the use of buccal swabs from healthy Hungarian volunteers, and then samples were purified by standard procedure. The amplification of the *rs1046322* and *rs9457* SNPs was performed by allele-specific PCR for ultrafast molecular haplotype analysis. PCR amplification reactions were performed in a total volume of 10 μL, which were carried out with approximately 4 ng DNA template, 0.5 U HotStarTaq DNA polymerase (Qiagen, Valencia, CA, USA), 200 μM dATP, dCTP, dGTP, dTTP, and 1 μM of each outer oligonucleotide primer (Sigma Genosys, Woodlands, TX, USA). The sequences of the primers were designed by the Oligo 5.0 software (Molecular Biology Insights, Cascade, CO, USA). Thermocycling conditions were started at 95ºC for 15 min to activate the hotstart DNA polymerase enzyme. This was followed by 40 cycles of denaturation (94ºC for 30 s), annealing (55ºC for 30 s), and then extension (72ºC for 1 min). The last procedure of the PCR was a final extension at 72ºC for 10 min after that the PCR samples were stored at 8ºC. After that, the PCR products were analyzed with agarose gel electrophoresis.

3.3.2. Analysis of PCR-Product by Agarose Gel Electrophoresis

DNA molecular mass marker (size range of 100 – 1 000 bp, Thermo Fisher Scientific) and 20 ng of PCR samples were electrophoresed for 45 min at 100V. Agarose gels comprised 2% agarose in 1× TAE electrophoresis buffer containing 0.5 μg/ml ethidium-bromide as an intercalating dye. The separated DNA bands were detected in a UV light box (Gel-Doc XR System, Bio-Rad, Hercules, CA, USA).

3.3.3. Analysis of PCR-Product by CGE

Rapid CGE analysis of the PCR-fragments was also accomplished by the DNA-CE instrument (BiOptic) with an 11 cm effective length (total length: 15 cm) fused silica capillary (internal diameter: 75 μm). To improve precision, two bracketing standard was used (20 bp and 5000 bp, BiOptic).

Samples were injected electrokinetically, and separations were carried out at ambient temperature by applying 6 kV (400 V/cm) electric potential. Data analysis was also performed using the Q-Analyzer software package (BiOptic).

3.4. Immune Response against Neoglycoproteins

3.4.1. Synthesis of Neoglycoproteins

Acetylation step: All the OH groups of maltose were acetylated by acetic anhydride and sodium acetate to yield crystalline compound, *2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-O-acetyl-β-D-glucopyranose*. ***Deprotection step:*** The anomeric OH group of maltose octaacetate was deprotected by hydrazine-acetate, and then *2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranose* compound was converted into the corresponding trichloroacetimidate donor. The anomeric configuration (α, β) was verified by NMR. ***Spacer addition step:*** The trichloroacetimidate donor was reacted with the 7-(1,3-dioxan-2-yl)-heptan-1-ol spacer to form a protected maltose with the formyl-heptyl spacer. ***Conjugation step:*** After removing the protecting acetal- and acetyl groups, the functionalized maltose was linked to the lysine ε-amino groups of BSA to form the Schiff base, which was consequently reduced with $NaCNBH_3$ to obtain a stable synthetized neoglycoprotein that served as synthetic carbohydrate hapten of the immunogen. The coupling reactions were performed for different rations hapten-spacer/BSA, so different neoglycoproteins were obtained, i.e. BSA-32-maltose, BSA-50-maltose, BSA-59-maltose, and BSA-66-maltose.

3.4.2. Analysis of Neoglycoproteins by SDS-CGE and MALDI-TOF MS

For purity and homogeneity, the reaction products were first analyzed by P/ACE MDQ system (SCIEX) connecting a 75 μm i.d. (360 μm o.d.) bare fused silica capillary with an effective separation length of 10 cm (total length: 30 cm). For all experiments a commercially available SDS-MW gel buffer (SCIEX) was used at a separation temperature by applying 15 kV (500 V/cm) electric field strength. MALDI-TOF MS based molecular mass assessment of the synthesized neoglycoproteins was carried out in positive reflectron mode using a BIFLEX III mass spectrometer (Bruker) with delayed-ion extraction (multiple >100 laser shots with N_2 laser (337 nm) using 19 kV accelerating and 20 kV reflectron voltage).

3.4.3. Analysis of Neoglycoproteins with ELISA

After the immunization steps (BALB/c mouse, ♀) with both the lowest (32 units/mol) and highest (66 units/mol) amount of sugar-incorporated synthetic neoglycoproteins as immunogens (50 µg/mL in PBS, pH 7.4), the samples were investigated by ELISA. Briefly, the immunoassay plates were coated with synthetic neoglycoproteins, and then incubated at 37ºC for 1 h. In order to detect the polyclonal response, a total of 10 µg/mL and a twofold serial dilution from 1.25 to 0.08 µg/mL of immunogen coatings were used, and 0.2 µg/mL BSA and synthetic neoglycoprotein as immunogen coatings were prepared for the experiments, which were done in the presence of inhibitors. After washing twice the plates with PBS-TWEEN wash buffer, the remaining protein-binding sites were blocked by the addition of PVP-blocking buffer and then incubated at 37ºC for 30 min. After washing twice the plates with wash buffer, diluted immune sera (1 000 – 128 000×) were added to the reactions followed by incubation at 37ºC for 1 h to check the polyclonal antibody response. This was followed by washing the plates four times with wash buffer, before 8000-fold diluted horseradish peroxidase conjugated secondary antibody was added to each well, and then incubated at 37ºC for 30 min. After washing again four times the plates with wash buffer, 3,3',5,5'-tetramethylbenzidine substrate solution was added to each well in order to measure the bound antibody. The estimated incubation times for the enzyme-substrate reaction ranged from 10 to 15 min, after which a stop solution was added to the reaction and the plates were interrogated at 450 nm in a Multiscan Ascant reader (Thermo Fisher Scientific, Hudson, NH, USA).

3.5. High-Performance CGE Analysis of Complex Carbohydrates

3.5.1. Sample Preparation for CGE

Glycoprotein digestions, i.e. 50 µg of IgG and RNase B, were performed using the GlykoPrep Digestion Module according to the manufacturer's protocol (Prozyme). After the centrifugation step, the maltodextrin ladder, dried sugars, D-(+)-maltose monohydrate and carbohydrate standards were labeled with ANTS-fluorescent dye, and then incubated at 37°C overnight. After that, the unreacted fluorescent dye was subsequently removed by Cleanup cartridges according to the protocol of the manufacturer (Prozyme). In CGE-LEDIF separation, ANTS-labeled-maltose (ANTS-G2) was used as lower bracketing standard and 5-ANSA-labeled maltose (ANSA-G2) was applied as the upper bracketing standard.

3.5.2. N-Glycan Separation by CGE

Rapid CGE separations of the ANTS-labeled *N*-glycans were performed by a single-channel Glycan Analyzer (BiOptic) instrument containing a 50 µm i.d. (360 µm o.d.) N-CHO capillary with an effective separation length of 11.5 cm (total length: 15.5 cm). The instrument contained an LED source of 350 – 390 nm range to match the ANTS excitation/emission spectra. For all experiments, a commercially available gel-buffer was used (BiOptic) at an ambient temperature by applying 6 kV (387 V/cm) electric potential. To improve precision, two bracketing standard was used. Migration times were converted to relative migration times using the bracketing standard boundaries. After that, normalized migration times were converted to GU values using a time based standardization against a maltooligosaccharide ladder.

3.6. Study of the Activation Energy by CGE

3.6.1. Sample Preparation for CGE

All endoglycosidase digestions (hIgG) were performed in sodium-bicarbonate solution, pH 7.0, and then reduced with DTT for 15 min at 65ºC and alkylated with IAA for 30 min at 37ºC in dark. After incubation, PNGase F (Prozyme) was added to the solution and incubated overnight at 37ºC. The released *N*-linked glycans were partitioned with CleanSeq magnetic beads (SCIEX). The maltooligosaccharide ladder, the maltose internal standard and the purified sugars were fluorescently labeled via reductive amination by the addition of APTS fluorescent dye, and then incubated for 1 h at 50ºC. After the labeling reaction, the unreacted fluorescent dye was removed with the CleanSeq magnetic beads (SCIEX). Viscosity measurements of the buffer systems were made at 20 − 50ºC on an AR 550 model rheometer (TA Instruments, New Castle, Germany) equipped with a 60 mm diameter, 2° angle steel cone geometry and a built-in Peltier heating/cooling plate.

3.6.2. CGE-based Separation of Linear and Branched Carbohydrates

Rapid CGE separations of the APTS-labeled oligosaccharides were performed in a PA 800 Plus Pharmaceutical Analysis System (SCIEX) containing a N-CHO capillary (50 µm i.d., 31 cm total, 21 cm effective length) using a laser based fluorescent detector. To alleviate for any variations caused by temperature changes, the CGE instrument was controlled by a liquid cooling system. All samples were analyzed at 20 – 50ºC using 25 mM lithium acetate buffer containing (pH: 4.75) i) no additives, ii) 0 – 60% ethylene glycol, iii) 0 – 3.0% linear polyacrylamide and iv) 0.4 % 300

kDa polyethylene oxide. The applied electric field strengths were 12 400 V (400 V/cm, reverse polarity). For migration time correction and normalization, APTS-labeled maltose (APTS-G2) was co-injected with each sample. Normalized migration times and GU values were calculated as mentioned above. Hydrodynamic volumes of relevant (DP 7-12) and major IgG *N*-glycans (FA2, FA2[6]G1, FA2[3]G1, and FA2G2) were calculated using TURBOMOLE 6.3 quantum chemical program package and COSMOtherm suite (COSMOlogic GmbH, Leverkusen, Germany).

4. RESULTS

4.1. Large-Scale gDNA Analysis by CGE and slab gel electrophoresis

In the first part of this study, an efficient micoball ended fiber optic based CGE-LEDIF detection system was introduced for large-scale genomic DNA (gDNA) analysis, also utilizing a single-channel separation capillary. To assess the suitability of this novel system, the degradation level of a close to a thousand gDNA samples were analyzed. The capability to quickly distinguish between intact and degraded gDNA samples was a crucial step for downstream quantitative PCR fragment analysis. In CGE analysis, a DNA molecular mass ladder was used in the base pair range of 75 – 20 000 bp for fragment size determination. Non-degraded intact gDNA sample showed a large peak pattern at the 2 000 base pair range of the separation area, while the variously degraded gDNA samples featured patterns at the lower base pair range of several hundreds, i.e. strongly suggesting sample degradation. Compared to CGE analysis, several degraded DNA bands were not visible on 2% agarose slab gel, while all degraded DNA peak patterns were clearly detected by CGE. In addition, the migration time reproducibility of this CGE system was determined by a 10-run repeatability study using 100 bp Plus DNA mass ladder (size range of 100 – 3 000 bp). The average migration times of the DNA mass ladder were between 88.22 and 143.22 seconds. The average percent RSD of the migration times were between 0.5118 – 0.7477%, which exhibited an excellent reproducibility.

4.2. Molecular Haplotype Analysis

In the second part of this study, simultaneous multiple genotyping of two adjacent putative miRNA-binding SNPs in the WFS1 gene was introduced by an efficient double-tube allele-specific amplification method in conjunction with a novel CGE-LEDIF detection system to investigate the resulting DNA fragments.

Direct haplotype determination by allele-specific amplification, i.e. molecular haplotyping, offers fast and reliable genotyping data of many single nucleotide polymorphisms (SNPs) in a single tube followed by CGE analysis. In this molecular haplotype study, the *rs9457* and *rs1046322* SNPs are presumably miR-SNPs, which are located in the WFS1 gene 3' UTR. For the chromosomal localization, a sense *rs1046322*- and an antisense *rs9457*-specific primer were used in the amplification reaction. One reaction mixture examined the presence of one allele at each loci as well as one haplotype combination, consequently two reaction mixtures were necessary to determine the genotype and the haplotype. To determine the size of the double allele-specific amplicons in the case of both haplotypes, CGE and conventional techniques were used. The CGE traces of the PCR fragments created by the haplotyping protocol. For fragment size assessment, the DNA mass ladder was extended with fragments in the size range of 50 – 3 000 bp. To obtain high fragment sizing accuracy, the multiplex PCR samples were co-injected with the lower and upper alignment markers (20 bp and 5000 bp). The CGE-LEDIF separations of three dsDNA fragments were represented from the reaction mixture-I with calculated sizes of 454, 500 and 583 bp fragments, corresponding to 437, 488 and 541 bp of the actual PCRs. The CGE-LEDIF separation of four dsDNA fragments were represented from the reaction mixture-II with calculated sizes of 399, 457, 504 and 591 bp fragments, corresponding to 384, 437, 488 and 541 bp of the actual PCRs (with better than 95% average accuracy). The bp accuracy of each DNA fragment was analyzed by the Q-Analyzer software package with the accuracy range of 2.4 – 9.2%.

To determine the detection limit of the system, the 576 bp WFS1 PCR fragment was used. When the detector linearity analysis was performed with the water diluted samples, the linear detection range was quite narrow, i.e. 1.5 orders of magnitude, probably due to the effect of field amplification. Therefore, the detection linearity study was accomplished by using a dilution series in sample buffer in that case a linear detector response was obtained in a large interval of 0.08 – 10.0 ng/µL with an R^2= 0.9997. Injection from water diluted samples resulted in much larger sample intake, because the buffer co-ions did not compete with the sample molecules, resulting in excellent LOD. On the other hand, measurements of the sample concentration were more precise from buffer diluted samples. The LOD of the system was 0.08 ng/µL for samples in buffer as well as 0.002 ng/µL for samples in water.

4.3. Immune Response against Carbohydrate Antigens

Antibody development against carbohydrates is of increasing importance, because carbohydrate antigens play a crucial role in biomarker research and discovery. For detecting carbohydrate antigens, the anti-carbohydrate antibodies have been utilized as diagnostic markers in several diseases as well as it may occur as neoglycoprotein-based antibacterial vaccines or neoglycoprotein-based antitumor vaccines for cancer. The synthesis of neoglycoproteins particularly incorporates random or defined coupling sites on the surface of the carrier protein, which is covalently modified with glycans via their reducing end or functionalized glycan bearing spacer arms.

In the third part of this study, a simple sugar-specific antigen (maltose) was conjugated to a carrier protein (namely BSA) by reductive amination. BSA was chosen as a carrier protein since in its natural form it voids glycosylation. To conserve the intact annular maltose structure, an aglycone (formyl-heptyl) spacer was utilized for the synthesis of neoglycoproteins. Neoglycoproteins were synthesized under controlled conditions with different numbers of oligomeric/dimeric maltose molecules (between 32 and 66 units/mol) linked to the lysine ε-amino residues of BSA. After modifying BSA with many different sugars, the number of conjugated sites was determined by MALDI-TOF mass spectrometry, having 32, 50, 59, and 66 units/mol maltose units. In addition, the reaction products were analyzed by SDS-CGE for purity and homogeneity. In this analysis, a decrease was observed in signal intensity of the glycoconjugates with increasing sugar unit content and the longer migration times during the separation. The former was due to the effect of the larger molecular mass of the conjugates with the attached maltose residues and the concomitantly suppressed SDS binding to these hydrophilic patches, both causing migration time increase. The decreasing signal intensity of the conjugate (and the increasing signal intensity of the unconjugated free dye) with the increasing maltose content was due to the reduced number of available conjugation sites for the fluorescent dye, i.e. remaining free amino residues on BSA. The incorporation level of sugars increased from 32 to 66 units by increasing the molar ratio of the maltose to BSA. This result was surprising, because BSA only contained 60 free amino groups but can be explained by the studies of *Schwartz* and co-workers, who demonstrated that tertiary amines can be also formed during the reductive amination reaction possessing two sugar structures, consequently increasing the maximum number of conjugated glycans beyond 60.

The neoglycoproteins were then evaluated by immunization, and then the polyclonal antibody response was analyzed by ELISA as evidence for the presence of sugar-containing epitope-specific antibodies. First, the BALB/c mice were immunized using carbohydrate antigens with low and high sugar incorporation levels (32 and 66 maltose units/BSA). In mice, immunized with the 32 maltose units/BSA, a rather unified response was observed towards the different antigens. In mice, immunized with the 66 maltose unit/BSA, the IgG response was more intense towards the modified antigens and was distinguishable to that of the significantly weaker carrier specific response. When the experiment was monitored under immunogen limiting conditions, this difference was even more pronounced. Thus, a more significant antibody response could be observed against neoglycoproteins than against the carrier proteins in the presence of limited amount of immunogen. The recognition of the carrier and the glycan modified BSA by the immune sera of some mice suggested the presence of antibody populations specific for the neoglycoproteins.

On the other hand, no IgG antibody response was detected in the presence of free maltose and other sugars such as glucose, isomaltose, lactose, galactose and maltodextrin. This result suggests that the synthesized glycoconjugates contained specific epitopes, which were unique to the presence of the defined number of conjugated maltose units per BSA molecules.

4.4. Generation of an ANTS-labeled *N*-Glycan Database for CGE Analysis of Carbohydrates

In this part of the work, an 8-aminonaphtalene-1,3,6-trisulfonate (ANTS)-labeled *N*-glycan database was developed for the LED-induced fluorescent (LEDIF) CGE system. The establishment of ANTS-labeled *N*-glycan database includes 25 oligosaccharides of mostly biopharmaceutical interest, such as for *N*-glycosylation profiling of therapeutic antibodies. For the generation of this comprehensive *N*-glycan database, the individual glycan standards were labeled via reductive amination using the ANTS labeling reagent and then separated by CGE. Finally, normalized migration times and GU values were calculated as mentioned above. To support the highest possible precision of migration time measurement, a lower (ANTS-G2) and a higher (ANSA-G2) bracketing standard were co-injected with the sample in all CGE separations. This database represents the abbreviated names of the individual glycan standards with their graphic representation, the symbol nomenclature, the precise molecular mass and the glucose unit (GU) values with the corresponding standard deviations (SD).

The resulted GU values can be applied as reference values for rapid structural elucidation of unknown glycan samples as explained below in reference to human IgG and bovine pancreatic RNase B. For the validation of this comprehensive *N*-glycan database, the generated GU values in this database were verified with *N*-glycans released from glycoproteins (human IgG and bovine RNase B). Briefly, glycoproteins were first released by the endoglycosidase PNGase F, and then the liberated sugars were labeled via reductive amination using the labeling reagents. After that, the ANTS-labeled *N*-glycans were separated by CGE. Very little differences can be observed between the GU values of the corresponding glycoprotein derived glycan peaks and the individual standards (hIgG: <0.14 GU, RNase B: <0.13 GU). CGE analysis times were around 200 s.

The corresponding database values were in good agreement with the results of glycoprotein derived glycans. For rapid assessment of *N*-glycan profiles, these 25 database entries will prove useful for fellow separationists to analyze their glycan profiles.

4.5. Effect of Separation Temperature and Background Electrolyte Composition on Structure Specific Glycan Migration in CGE

In the final section of this study, the activation energy (E_a) concept with electromigration of linear (maltooligosaccharides) and branched oligosaccharides (sialylated, neutral, and core fucosylated biantennary IgG *N*-glycans) was analyzed by CGE-LIF using viscosity modifier (0 − 60% ethylene glycol) or polymeric additive (0 − 3.0% linear polyacrylamide, 0.4% 300 kDa polyethylene oxide) containing BGE in the temperature range of 20 − 50ºC.

The polymeric additive was used below and above its entanglement threshold concentration (1.5%), representing both diluted polymer solution and entangled polymer network states. Interestingly, a clear breakpoint was observed around maltoheptaose (D7), creating two conformations, i.e. random coil (DP <7) and elongated helical (DP >7) structures. For oligosaccharides shorter than DP 7 the E_a values increased by the rate of 212 J/mol per glucose unit, while the E_a values only increased with the rate of 68 J/mol per glucose unit for oligosaccharides longer than DP 7. This conformation change was considered to be the reason for this 144 J/mol per glucose unit transition in the E_a values. However, the shorter oligosaccharides (DP <7) may have their less hydrophilic side exposed (no helix yet) that might interact with the –(CH_2-CH(CO-NH_2)-CH_2)-LPA chains in the background electrolytes, resulting in higher E_a

requirement. In case of higher polymeric additive concentrations (2.0 – 3.0%), shallower E_a slopes were observed for both segments, because the polymer network should be more entangled, therefore less non-networked LPA chains are available to interact with the solute molecules. At the entire range of 0 – 3.0 % polymeric additive concentration, the slope values were positive, suggesting no analyte and/or no network deformation. In addition, under practically equal viscosity conditions, the average E_a requirement for oligosaccharides of DP 2–7 for the 10% ethylene glycol and 2.0% linear polyacrylamide containing gel-buffers were 72 and 212 J/mol. Please note that the viscosity of 2% LPA solution was very similar to the 10% EG containing BGE at 20ºC and 50ºC, i.e. 2% LPA (20ºC) = 1.30 mPa.s; 2% LPA (50ºC) = 0.82 mPa.s and 10% EG (20ºC) = 1.28 mPa.s; 10% EG (50ºC) = 0.74 mPa.s.

This dramatic difference (140 J/mol) was probably due to solute-matrix interaction effects. For oligosaccharides longer than (DP> 7), the E_a values were very similar that of in the monomeric viscosity modifier containing gel-buffer (68 versus 72 J/mol), suggesting that the closure of the helical structure apparently alleviated any matrix interactions. On the other hand, if background electrolytes containing no or only monomeric (ethylene glycol) additives were used, the GU values of the branched biantennary IgG *N*-glycans decreased with increasing separation temperature. However, with background electrolytes containing polymeric additives (linear polyacrylamide, polyethylene oxide) the GU values increased with increasing temperature, probably due to possible solute-network interactions or deformations effects in addition to the influence of viscosity. While in the presence of additive-free background electrolyte the corresponding average GU values shift of the branched glycans were -0.89 GU, this value changed to -0.39, +0.23 and +0.38 GU unit by the addition of 10% EG, 2% LPA and 0.4% PEO, respectively, in the temperature range of 20 − 50ºC.

To calculate the E_a values, the Arrhenius diagrams were plotted for all additive types. After that, the E_a vs hydrodynamic volume diagrams were plotted and the E_a changes were derived for the both the linear and branched structures as 0.3 J/mol per A^3 and 0.9 J/mol per A^3, 0.4 J/mol and 1 J/mol per A^3, as well as 0.06 J/mol and 0.3 J/mol per A^3 for the background electrolytes containing EG, LPA and PEO. In the presence of the monomeric (ethylene glycol) additive, the E_a requirement was presumable associated with the passage of the increasing size analyte molecules through the higher viscosity background electrolyte. In the instance of polymeric additive, in addition to the viscosity component, other physical interactions between the analyte molecules and the polymer network and/or deformation effects should also be considered.

5. DISCUSSION

Large-Scale Genomic DNA Analysis by CGE

Currently, most bioanalytical laboratories still utilize manual slab gel based electrophoresis techniques, which have routinely been used for checking DNA properties. However, these methods are time consuming and labor intensive, also requiring improvements in terms of resolving power and analysis throughput. Combining the sensitivity of fluorescence detection with the variability of replaceable polymer sieving and the opportunity of automation in both sample handling and data acquisition, CGE has become an attractive alternative to traditional slab gel electrophoresis. In the first part of this study, the aim was to introduce a novel single-channel CGE system with LED-induced fluorescence detection utilizing a pen-shaped capillary cartridge for automatic separation of samples from a 96-well plate. The separation performance of this instrument was demonstrated by rapid and large-scale purity analysis of a thousand gDNA samples, exhibiting excellent migration time reproducibility (RSD <0.75%) and detection limit of ~0.1 ng/μL in comparison to manual agarose slab gel electrophoresis. In addition, the instrument quickly distinguished between degraded and intact gDNA samples, thus provided crucial information if they could be used for downstream quantitative PCR processing where high-quality intact gDNA was the key.

Molecular Haplotype Analysis by CGE

Wolframin (WFS1) is a transmembrane protein in the endoplasmic reticulum, which is generated at higher levels in pancreatic beta cells and specific neurons in the central nervous system. Genetic variations in the WFS1 gene have been described to be associated with the Wolfram syndrome or type 2 diabetes mellitus. Simultaneous study of multiple polymorphisms (i.e. haplotyping) is getting more and more attention in the analysis of genetic variations of complex diseases. Direct molecular haplotyping is of particular importance in the case of double heterozygote samples, since in these instances the haplotype structure cannot be constructed simply based on genotype data. Our group successfully reported on genotyping and haplotyping in the dopamine D4 receptor gene by CGE-LIF separations. However, currently, there are no sensitive and powerful platforms in most bio-analytical laboratories for genotype and haplotype analysis by CGE-LEDIF system. Thus in the second part of this study, the aim was to elaborate an efficient double-tube allele-specific PCR-based approach in conjunction with ultrafast CGE-LEDIF system for direct haplotyping of the SNPs in two important miRNA-binding sites (*rs1046322* and *rs9457*) in the WFS1 gene.

In addition, the separation performance was demonstrated by ultrafast (<240 s) and accurate (2.4 – 9.2%) sizing analysis of multiplex PCR samples, exhibiting excellent detector linearity (R^2=0.9997) with a dynamic quantitation range of 0.08–10.0 ng/μL. Moreover, the detection limit was 0.002 ng/μL using field amplified injection from water diluted samples. In conclusion, this CGE-LEDIF system offers a sensitive and easy to use bio-analytical tool for automated haplotyping of a large number samples in clinical settings.

Immune Response against Carbohydrate Antigens

Today, antiglycan antibody development against carbohydrate antigens is of increasing importance since glycosylation is recognized as a significant player in biomarker research and discovery. Therefore, the aim was to investigate polyclonal antibody response for newly synthesized maltose-BSA conjugate neoglycoproteins. In the third part of this study, selective antibody binding was demonstrated to the synthesized carbohydrate antigens with different glycosylation degrees (low and high) suggesting the possible use of this approach to generate antiglycan antibodies. Moreover, the polyclonal antibody response was not inhibited by maltose or other simple carbohydrates such as glucose, isomaltose, lactose, galactose, and maltodextrin to confirm presence of the neoglycoprotein-specific antibodies. It remains to be determined, however, whether the new immunogenic epitopes included the sugar component or merely represented conformational changes of the core polypeptide chain induced by the sugar conjugation process. To the best of my knowledge there is no report suggesting that the core protein structure was changed by glycosylation changes or some polyclonal antibody response to glycans with detectable specificity directed strictly to the sugar moiety. In the future this elaborated method may be utilized for conjugation of complex disease specific sugar structures to carriers in order to generate monoclonal antibody libraries.

Generation of an ANTS-labeled N-Glycan Database for CGE Analysis

There is an increasing trend to develop therapeutic glycoproteins, such as monoclonal antibodies, which require sensitive and high-resolution bioanalytical techniques for comprehensive carbohydrate characterization. Protein derived glycans in most instance have a large variety of structural diversity such as positional and/or linkage isomers, which calls for the need of state-of-the-art instrumentation for their analysis. Thus, high-performance analytical techniques including CGE is a well-established tool, which plays a significant role in the structural elucidation of glycosylation in the biomedical and pharmaceutical fields. On the other hand, the time consuming

carbohydrates sequencing step in most *N*-glycan profiling studies can be avoided with the use of a comprehensive glycan glucose unit (GU) database, which is specific for a given instrument and gel-buffer system. Our laboratory reported the successful establishment of an APTS-labeled *N*-glycan database released from human serum polyclonal IgG using LIF detection. However, currently there are no well-established, automated, and high-throughput bioanalytical platforms for CGE-LEDIF systems to provide rapid analysis of biopharmaceutical or biotechnology samples of interest without the need of additional carbohydrate sequencing steps. Therefore, the aim was to introduce an initial version of a novel GU database for ANTS-labeled *N*-glycans by CGE-LEDIF detection. Comparison of the normalized migration times of the peaks of interest of glycoprotein derived *N*-glycans with carbohydrate standards with known GU values in this database poses a simple and effective way for rapid structural assessment. This database provided 25 *N*-linked glycan structures of mostly biopharmaceutical interest such as for *N*-glycosylation profiling of therapeutic antibodies with rapid (around 200 s) CGE separation times. The validation of the generated GU values in the database was verified with *N*-glycans released from human IgG and bovine ribonuclease B, which showed very little differences between the database values and measured values (hIgG: <0.14 GU, RNase B: <0.13 GU). On the other hand, glycans are structurally diverse, therefore in some rare instances there are possible co-migrations of species, in which cases exoglycosidase enzyme array based carbohydrate sequencing can be applied for correct structural elucidation. In the future this novel application may provide a broadly applicable bio-analytical tool for rapid glycan analysis of biotechnology and clinical samples.

Effect of Separation Temperature and Background Electrolyte Composition on Structure Specific Glycan Migration in CGE

For the physicochemical characterization of biopolymers, CGE has been extensively utilized using cross-linked gels and/or linear polymer sieving matrices. However, today almost exclusively linear polymer gels are used in CGE due to the difficulties of working with cross-linked sieving matrices in narrow bore capillary tubings. On the other hand, with the use of linear polymer gels, network dynamics should be considered both below and above their entanglement. In addition, the activation energy concept is often used to investigate possible temperature induced deformations affecting the analyte, the network, or both.

Therefore, in the last part of this study, the aim was to investigate the effect of separation temperature on the differential electromigration shift between linear (maltooligosaccharides) and

branched (sialylated, neutral and core fucosylated biantennary IgG glycans) carbohydrates in narrow bore capillaries in the range of 20 − 50 °C. To understand the structure specific electrophoretic migration of the different sugar oligomers, the activation energy concept was used in this study. In addition, viscosity modifiers (e.g., 0 − 60% ethylene glycol) and/or polymer additives (e.g., 0 − 3% linear polyacrylamide and 0.4% polyethylene oxide) were added to the background electrolytes in order to investigate this phenomenon. The results have shown that glucose unit value shift were observed with increasing temperature between the linear and branched sugar structures caused by the temperature-dependent activation energy requirement in order to migrate through the polymer network. Therefore, this emphasizes the high importance of tight temperature control during glycan analysis by capillary electrophoresis if glucose unit values form existing databases are used for structural elucidation.

6. SUMMARY

In the first part of this study, the design of a single capillary CGE system was introduced with a pen-shaped compact capillary cartridge comprising a novel microball ended fiber optic-based LED-induced fluorescence detection setting. This automated system offered a good and easy-to-use alternative to labor intensive slab gel electrophoresis systems, also featured excellent detection sensitivity, high-resolving power and rapid analysis times for quantitative or qualitative analysis of biopolymers. The separation performance of this instrument was demonstrated by rapid and large-scale purity analysis of close to a thousand gDNA samples, exhibiting excellent migration time reproducibility (RSD <0.75%).

CGE-LEDIF-based method was also applied to the analysis of multiplex PCR amplification for genotyping and haplotyping of two important, adjacent miRNA-binding sites (*rs1046322* and *rs9457*) in the WFS1 gene. The separation performance was also demonstrated by ultrafast (<240 s) and accurate (2.4 – 9.2%) sizing analysis of multiplex PCR samples, exhibiting excellent detector linearity (R^2= 0.9997) with a dynamic quantitation range of 0.08–10.0 ng/μL. The LOD of the system was 0.08 ng/μL for samples in buffer and 0.002 ng/μL for samples in water, this latter was due to the field amplified injection effect.

In the third part of this study, the analysis and polyclonal antibody response for newly synthesized maltose-BSA conjugates was described. First of all, a simple carbohydrate, maltose, was linked to BSA by reductive amination.

To conserve the intact annular maltose structure, an aglycone spacer was utilized for the synthesis of neoglycoproteins. The synthesized neoglycoproteins were analyzed by SDS-CGE and the number of conjugated maltose residues was determined by MALDI-TOF MS. The carbohydrate antigens were then evaluated by immunization of BALB/c mice and the polyclonal antibody response was analyzed by ELISA as evidence for the presence of sugar-containing epitope-specific antibodies. Selective antibody binding was demonstrated to the synthesized neoglycoproteins with different (low and high) glycosylation degrees offering the possible use of this approach to generate antibodies. In addition, the polyclonal antibody response to these neo-epitopes was not inhibitable by maltose or other simple and oligomeric sugars like glucose, isomaltose, lactose, galactose, and maltodextrin. It should be emphasized that no report was found in the literature suggesting that the polyclonal antibodies response would be generated against core protein structure (BSA).

In the fourth part of this study, the establishment of a novel ANTS-labeled *N*-glycan database was introduced for rapid (around 200 s) CGE analysis of complex *N*-linked carbohydrates. The validation of the generated GU values in this database was accomplished by the use of *N*-glycans released from human IgG and bovine pancreatic RNase B. The corresponding database values were in good agreement with the results of glycoprotein derived glycans. For rapid assessment of *N*-glycan profiles, these 25 database entries will prove useful for fellow separationists to analyze their glycan profiles.

In the final section of this study, the activation energy concept with electromigration of linear and branched oligosaccharides was investigated in CGE using viscosity modifier (ethylene glycol) or polymeric additive (linear polyacrylamide, polyethylene oxide) containing BGE in the temperature range of 20 – 50ºC. The relationship between the E_a and the DP of linear malto-oligosaccharides in the range of 1-15 was closely scrutinized with special respect to the temperature-dependent GU values shift of branched *N*-glycans. The GU value shifts were probably caused by the temperature-dependent E_a requirement for the different structures (linear vs branched), therefore this emphasizes the high importance of tight temperature control during glycan analysis by CE if GU values form existing databases are used for structural elucidation.

7. NEW RESULTS, FINDINGS AND THEIR SIGNIFICANCE

- In this study, a single capillary CGE system was introduced with a LED-induced fluorescence detection setting. Moreover, an aminonaphtalene trisulfonate (ANTS) labeled novel *N*-glycan database library was established for rapid structural elucidation of complex *N*-linked carbohydrates. Currently, this *N*-linked glycan database contains 25 oligosaccharide structures.
- Using the glycan mobility results, the activation energy (E_a) concept with the electromigration of different oligosaccharides was investigated in CGE at various temperatures and with different background electrolyte additives. It was found that the electrophoretic mobility of maltooligosaccharides depended on types of additives under practically equal viscosity conditions, due to their E_a requirement difference.
- A clear breakpoint was observed around maltoheptaose (DP 7) using polymeric additive containing BGE due to conformation differences, i.e. random coil at DP <7 and elongated helical structures at DP >7, suggested by the E_a values increase by the rate of 212 J/mol per glucose unit for oligosaccharides shorter than DP 7, while the E_a values only increased with the rate of 68 J/mol per glucose unit for oligosaccharides longer than DP 7. This conformation change was considered to be the reason for this 144 J/mol per glucose unit transition in the E_a values, probably because of the shorter oligosaccharides may have their less hydrophilic side exposed that might interact with the polymer chains in the background electrolyte, requiring higher E_a.
- The electrophoretic mobility of branched biantennary IgG *N*-glycans revealed temperature dependent changes in contrast to the linear sugar structure counterparts in the various background electrolytes. Thus, if background electrolyte containing no or only monomeric (ethylene glycol) additives were used, GU values of the branched *N*-glycans decreased with increasing temperature. However, with background electrolytes containing polymeric additives (linear polyacrylamide, polyethylene oxide) the GU values increased with increasing temperature. The increase was assumedly due to possible solute-network interactions or deformations effects in addition to the influence of viscosity.
- The GU value shifts were probably caused by the temperature-dependent E_a requirement for the different structures, emphasizing the high importance of tight temperature control during glycan analysis by CE if GU values form existing databases are used for structural elucidation.

8. PUBLICATIONS

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Subject: PhD Publikációs Lista

Candidate: Márta Kerékgyártó
Neptun ID: F10PDA
Doctoral School: Doctoral School of Molecular Medicine
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List of publications related to the dissertation

1. **Kerékgyártó, M.**, Járvás, G., Novák, L., Guttman, A.: Activation energy associated with the electromigration of oligosaccharides through viscosity modifier and polymeric additive containing background electrolytes.
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Total IF of journals (all publications): 34,273
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