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Abstract

We present in this study a novel strategy to drastically improve the detection sensitivity and peak capacity for capillary electrophoresis with laser induced fluorescent detection (CE-LIF) of glucose oligomers and released glycans. This is based on a new approach exploiting a polymer-free background electrolyte (BGE) for CE-LIF of glycans. The best performance in terms of sample stacking and suppression of electroosmotic flow (EOF) was found for a BGE composed of triethanolamine / citric acid and triethanolamine / acetic acid at elevated ionic strengths (IS up to 200 mM). Compared to the conventional protocols for CE-LIF of glucose-oligosaccharides and released glycans, our polymer-free strategy offered up to 5-fold improvement of detection sensitivity and visualization of higher degree of polymerization (DP) of glucose oligomers (18 vs 15). To further improve the detection sensitivity, a new electrokinetic preconcentration strategy via large volume sample stacking with electroosmotic modulation without having recourse to neutrally coated capillaries is proposed, offering a 200-fold signal enhancement. This approach is based on variation of the buffer's IS, rather than pH adjustment as in conventional methods, for EOF modulation or quasi-total reduction. This strategy allows selecting with high flexibility the best pH conditions to perform efficient preconcentration and separation. The new approach was demonstrated to be applicable for the analysis of N-linked oligosaccharides released from a model glycoprotein (Human Immunoglobulin G) and applied to map N-glycans from human serum for congenital disorders of glycosylation (CDG) diagnosis.

1. Introduction

Analysis of glycans released from glycoproteins plays an important role for quality control of therapeutic proteins and diagnostic purposes [1, 2]. Protein glycosylation is by far the most complex common post-translational modification, with more than half of all secretory and cellular proteins being glycosylated [3-5]. An altered glycosidic linkage or one truncated antenna in a glycan may alter the normal functioning and activity of glycoproteins. A variation of glycoform proportion of a given glycoprotein may also have biological consequences or be the result of a pathological state [6]. That is why glycoproteins represent a useful source of biomarkers for various illnesses like cancer and cardiovascular diseases [2]. Another typical example is congenital disorders of glycosylation (CDG), a family of multisystem genetic diseases caused by mutations in genes coding for proteins involved in the biosynthesis (CDG-I) or trimming/remodeling (CDG-II) of the oligosaccharide moieties of N-linked glycoconjugates [7]. Thus, CDG-I leaves some N-glycosylation sites unoccupied while CDG-II is associated with subtle structural changes of N-glycans with a whole array of isomers, leading to a great challenge for their separation and identification.

Until now, analysis of glycans have frequently been carried out with high-performance liquid chromatography with fluorescence detection (HPLC-FLD) [8], matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [9, 10], or liquid chromatography connected to electrospray ionization mass spectrometry (LC-ESI-MS) [7]. Capillary electrophoresis (CE) coupled with laser induced fluorescent (LIF) detection has become a staple in glycan separation, mostly for N-glycans [11, 12]. Prior to downstream analysis, sample preparation is required, especially when performing sensitive detection is the objective [13]. It stands for both small compounds and large biomolecules. In a standard protocol for glycans, glycans are first released from glycoproteins / peptides, enriched on a solid support (i.e., magnetic beads in most of the cases), then fluorescently labelled with a charged fluorophore (the most frequently used one being 8-aminopyrene-1,3,6-trisulfonic-acid (APTS) [14]) and eventually separated by CE-LIF. Normally, acidic background electrolytes (BGEs) containing different polymer/gel types and concentrations, typically polyethylene oxide (PEO) or polyethylene glycol (PEG), are used [15]. The CE-LIF conditions for glycan analysis have recently been established with the introduction of commercial kits, including polymer-containing buffers [16, 17]. Efforts to use gel-free BGEs for such purpose were also reported in the early 90's [18-20], but were then outnumbered by those that use gels and polymers in recent years.

So far, reported BGE compositions for CE-LIF of glycans normally employ inorganic and small ions (*e.g*., phosphate, borate, acetate, sodium, lithium) as well as inorganic acid and base (typically NaOH and HCl) for pH adjustment. From our viewpoint, such BGEs with low UV absorbing features are adapted for UV detection but the significant increase in their ionic strengths, which is required for improving analyte stacking and suppressing further the electroosmotic flow (EOF), is not trivial to achieve due to detrimental increase in electric currents and thus Joule heating. To further increase the detection sensitivity and peak capacity, the challenge is to find a BGE that simultaneously satisfies the criteria of efficient sample stacking (for increased glycans' signals), EOF suppression (for migration of large glycans counter the EOF to the detector) and tolerable current generation. A straightforward, cost-effective and coating-free CE-LIF approach that offers such features for glycan separation is therefore highly desirable. In the diagnostic context, there are still challenges for the detection of glycans used as biomarkers, mostly due to the low abundance of some plasma glycoproteins and unsatisfactory detection sensitivity. This therefore requires continued development to improve the CE-LIF performance towards the screening/diagnosis of aberrant-glycan-related pathologies. Efforts have been focused on glycan sample treatment (fluorescent labeling [21-23]) and glycan enrichment methods based on solid phase extraction [24, 25] or electrokinetic phenomena (notably isotachophoresis and large volume sample stacking) [26-29] prior to CE-LIF.

In this study, we report a novel strategy to significantly improve the performance of CE-LIF of fluorescently labelled glycans, using polymer-free buffers in conjunction with efficient analyte stacking and EOF suppression in fused silica capillaries. To demonstrate the applicability of our new approach, CE-LIF analyses of N-glycans released from a model glycoprotein (Human Immunoglobulin G) were carried out and the performance in terms of signal sensitivity and peak capacity was compared to currently used methods. The developed approach was applied for mapping of N-glycans released from human serum glycoproteins, serving for CDG screening.

2. Experimental

2.1. Chemicals, reagents and samples

All chemicals for preparation of buffers were of analytical or reagent grade and purchased from Sigma-Aldrich (Lyon, France). Reagents for preparation of glycans (denaturation, digestion, labeling solutions; malto-oligosaccharides (MD ladder), APTS dye, magnetic beads and G3 standard) were taken from the Fast Glycan Analysis and Labeling kit from SCIEX (Villebon sur Yvette, France). Human Immunoglobulin G (IgG), PNGase F and sodium cyanoborohydride ($NaBH_3CN$) were purchased from Sigma-Aldrich (Lyon, France). Maltose (G2) was obtained from Sigma-Aldrich and Maltohexose (G6) was obtained from Carbosynth Limited (Compton, UK). Beta-alanine (β-Ala), triethanolamine (TEOA), trimellitic acid, acetic acid (AcOH), citric acid (Cit) and lithium hydroxide monohydrate were used for preparation of BGE solutions. Serum samples from CDG individuals and healthy control were kindly provided by Dr. A. Bruneel (Bichat hospital, Paris, France).

2.2. Apparatus and Material

Method development was performed with a Beckman Coulter PA800+ and MDQ systems (SCIEX, Brea, CA) coupled with a LIF detector ($\lambda_{excitation}$: 488 nm, $\lambda_{emission}$: 520 nm). Instrument control and data acquisition were carried out by using the 32Karat ver 8.0 software (SCIEX). Fused silica capillaries of 50 µm i.d. and 375 µm o.d. from Polymicro (TSP050375, CM Scientific, Silsden, UK) were used for all CE experiments. Deionized water was purified using a Direct-Q3 UV purification system (Millipore, Milford, MA, USA) and the pH values of buffer solutions and samples were measured with a SevenCompact pH meter (Mettler Toledo, Schwerzenbach, Switzerland). Buffer ionic strength (IS) calculations were based on simulations with the computer program PhoeBus (Analis, Suarlée, Belgium). Additional information regarding the simulation by Phoebus program can be found in reference [30]. To maximize the accuracy for a defined buffer’s ionic strength, calculation was implemented with correction according to either Debye-Hückel equation (ionic strengths of 1-10 mmol/L), Güntelberg equation (ionic strengths of 10-60 mmol/L) or Davies equation (ionic strengths of 60-500 mmol/L). Deionized water was used for the preparation of all solutions.

2.3. Methods

2.3.1. Release and fluorescent labelling of glycoprotein derived-glycans

10 µL of human IgG (10 mg/mL in deionized water) was mixed with 5 µL of the denaturing solution containing 10 µL of D1 solution, 10 µL of D3 solution and 50 µL of D4 solution from the SCIEX kit and incubated at 70°C for 10 min. After denaturation, 12 µL of the digestion solution containing 5 µL of PNGase F in 12 µL of D4 was added and further

incubated for 60 minutes at 50°C. The released glycans were then labelled following the protocol described in [22]. Briefly, labelling reagents (3 µL of 40 mM APTS in 20% AcOH, 2 µL of 1 M $NaBH_3CN$) in THF, 4 µL of 20% AcOH and 6 µL of THF) were added into the sample vial and incubated for 60 min at 50°C with closed cap, and then for 60 min at 50°C via evaporative labelling with open cap.

For the preparation of N-glycans from human serum samples, the magnetic beads delivered in the SCIEX kit were used for sample purification. The tube containing 200 µL of resuspended magnetic beads was placed in a magnetic stand to allow the beads migrating to the magnet, then the storage buffer was removed. This step was performed every time to remove any liquid from the beads. 10 µL of 4-fold diluted serum sample was added onto the beads. 5 µL of denaturing solution was then added, mixed and incubated at room temperature for 7 min with the vial opened. 11 µL of digestion solution from Sciex kit was added to the mixture; and incubated at 60°C for 20 min in an open vial. 200 µL of acetonitrile was then added, mixed and incubated at room temperature for 1 min. The supernatant was removed, and 9 µL of 40 mM APTS in 20% acetic acid, 1 µL of NaCNBH3 and 1 µL of D4 from the SCIEX kit was added, followed by incubation at 60 °C for 20 min in an open vial.

2.3.2. Fluorescent labelling of standard oligosaccharides

The preparation of fluorescently labelled *MD* ladders was performed according to Reider *et al.* [22]. Briefly, 2 mg of oligosaccharide ladder was added in a 200 µL PCR tube, followed by addition of 4 µL of 40 mM APTS in 20% AcOH, 4 µL of 20% AcOH and 2 µL of 1M sodium cyanoborohydride ($NaBH_3CN$) in tetrahydrofuran (THF). The mixture was incubated under mixing at 70°C for 30 min with an open-capped vial. After the reaction, the sample was stocked in 100 µL deionized water, which was aliquoted and stored at -20°C. Further dilution of this stock solution was carried out before CE-LIF analysis.

2.3.3. CE-LIF of APTS-glycans and oligosaccharides

Analyses of the APTS-labeled N-glycans or the malto-oligosaccharide standards were carried out either with various BGEs detailed in Table 1 or using the SCIEX gel buffer system (BGE SCIEX). These separations were done using fused-silica capillaries of 50 µm i.d. with the total length (L_{tot}) of 60 cm, the effective length (L_{eff}) of 50 cm, under a separation voltage of -25 kV applied on the injection (inlet) side . The fused silica capillaries were preconditioned by rinsing with 0.1 M NaOH for 5 min, water for 3 min and the buffer of interest for 10 min prior to use.

Large-volume sample stacking with EOF modulation was implemented with BGE composed of TEOA / citric acid (IS of 150 mM, pH 4.75). The sample prepared in water was hydrodynamically filled to 100 % of the capillary volume (0.7 psi, 900 sec). A voltage of -25kV was applied on the injection (inlet) side to stack the target oligosaccharides / glycans. The preconcentration step was transitioned into the separation step without user intervention when the current stopped increase and reached a stable value.

3. Results and Discussion

3.1. Polymer-free BGEs for CE-LIF of APTS-labeled malto-oligosaccharides and glycans

3.1.1. Proposed principle

Our proposed approach, illustrated in Fig. 1A, is to push the ionic strengths (IS) of the BGE and concentrations to extremely high levels (IS up to 200 mM, which require concentrations up to a thousand mM) to allow high stacking of APTS-labelled glycans induced by the conductivity differences between the sample and buffer zones [31, 32]. At the same time, these conditions ensure suppression of EOF without recourse to any capillary coating via diminution of the electrical double layer thickness on the internal capillary surface [33, 34], while still maintaining tolerable electric currents. This EOF suppression is desirable to allow the anionic labeled glycans to migrate towards the detector. Whereas this was difficult with buffers containing inorganic ions, we expected it to be realizable with BGEs composed of large weakly charged molecules with low degrees of protonation / ionization. These molecules have indeed been used for preparation of BGEs for CE coupled with contactless conductivity detection. Their concentrations in this case are normally kept low (less than 50 mM in general) in order to minimize the generated background signal [31, 35].In our study, we targeted much higher buffer concentration ranges (up to a thousand mM) than typically reported. As the electric currents are directly related to the conductivity, which in turns links to the ion density of the BGE (*i.e.,* IS) and ions' electrophoretic mobilities (μ_{ep}), the very-slowly-migrating ions (ideally, μ_{ep} approaching zero) were anticipated to provide much lower currents than the conventional BGE constituents, even at very high IS and concentrations.

3.1.2. Performance of the polymer-free BGEs

To follow the logic described above, several BGEs that were designed by simulation using the Phoebus software to meet the aforementioned criteria were selected (Table 1). Four BGE candidates from the simulated ones were experimentally tested, including TEOA/ citric acid, TEOA / acetic acid, β-Ala / acetic acid and β-Ala / trimellitic acid. The EO mobilities obtained with these BGEs, measured using the method developed by Williams et Vigh [36], were 0.47×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$ for TEOA/ citric acid, 1.28×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$ for TEOA / acetic acid, 4.82×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$ for β-Ala / acetic acid and $4{,}72 \times 10^{-5}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$ for β-Ala /

trimellitic acid buffer, respectively. The EO mobility was reduced by up to 10 folds when using TEOA-based BGEs instead of β-Ala-based ones. Note that the EO mobilities achieved with neutral coatings are normally inferior to $1\times 10^{-5}\ cm^2\cdot V^{-1}\cdot s^{-1}$ [37, 38].These BGEs were used for the separation of the APTS-labeled MD ladder, and the performance was evaluated in terms of peak intensity and capacity. The lithium acetate buffer (LiOH/AcOH), a polymer-free BGE employed by many groups for CE-LIF of oligosaccharides [11, 15], was also tested as a reference. The others in the list (e.g., TEOA / gluconic acid, TEOA / sorbic acid, etc.) were not tested due to limited solubilities of the least soluble components in these buffers. Performance comparison for CE-LIF of the APTS-labelled oligosaccharide ladder with the 5 investigated BGEs is shown in Fig. 2. As can be seen, peak signals achieved with our 4 proposed BGEs (Fig. 2B-E) are all significantly higher than those obtained with LiOH/AcOH buffer (Fig. 2A). Particularly, the peak heights obtained with TEOA/Cit and TEOA/AcOH were up to 5-fold higher than those obtained with LiOH/AcOH BGE. Furthermore, the TEOA-based buffers could suppress EOF more efficiently, allowing detection of longer glucose oligomers, up to 12 and 17 glucose units (DP) for TEOA/AcOH (Fig. 2D) and TEOA/Cit (Fig. 2E), respectively, whereas only 6 DP peaks are found with LiOH/AcOH under the same CE conditions. In the case of 150 mM β-Ala/Trimellitic acid (Fig. 2B) and 150 mM β-Ala / AcOH (Fig. 2C), one can see that the signals were also approximately 5-fold higher than those from the reference LiOH/AcOH. The number of observed malto-oligosaccharides was, however, much lower than that for TEOA based buffers. The best performance in terms of peak heights and detected DP number was achieved with TEOA/AcOH and TEOA/Cit buffers. Table 2 further shows that the signal to noise ratios were approximately 10-times higher with TEOA-based buffers compared to the standard LiOH/AcOH one. Satisfactory repeatability for migration times (RSD % < 0.5 %) and peak areas (RSD < 5 %) was achieved for the investigated peaks with these buffers (Table 2). The overall results clearly show the superiority of TEOA/Cit BGE. The improved signals observed with our buffers are probably linked to the prolonged duration (Δt) to maintain the stacking effect. Indeed, good sample stacking in CE is not only dependent on the conductivity difference ($\Delta\sigma$) between the sample and electrolyte zones (similar to optimization of conductivity signals [31]), but also on the duration (Δt) to maintain such difference. With conventional BGE, the initial $\Delta\sigma$ is normally satisfied (*via* high μ_{ep} at low and moderate IS) at the starting moment, however, decreases when the fast-moving ions in the BGE zone penetrate into the sample zone (Kohlrausch regulating function [39, 40]). This stacking effect is on the other hand expected to reach the maximum for extremely concentrated slowly migrating BGE ions (see Fig. 1A), thanks to high $\Delta\sigma$ (*via* very high IS even with low μ_{ep}) and longer Δt (due to retardation of the ion penetration from the BGE into the sample zone). Furthermore, by using an extremely dense zone of large weakly charged ions in the BGE, the diffusion of stacked analytes may be hindered by the clusters or groups of electrolyte molecules [41]. This in turn can help “blocking” the stacked analytes more efficiently at the sample-BGE boundary for further peak height improvement.

The IS of this BGE was then further increased in order to achieve greater stacking effect, and thus gaining further improvement of peak signals for the APTS-labeled MD ladder (Fig 3). Oligosaccharide peaks obtained with TEOA/Cit BGE at IS of 200 mM were slightly higher (1.3 - 1.5x improvement) compared to those obtained with 150 mM IS. Interestingly, the EOF was slightly higher with the 200 mM BGE with some retardation of the oligosaccharide peaks, which was probably due to some increase in pH. Indeed, when working with very concentrated solutions, their experimentally measured pH values were found slightly shifted from the simulated ones. In the case of TEOA/Cit BGE at IS of 200 mM, the measured pH was 5.05 instead of 4.75 as simulated. This can refer to a similar situation when measuring the pH values of very salty solutions, where the aqueous proton (H^+) concentration is different from its activity, leading to a shift of pH value that reflects the solution H^+ activity. Consequently, fewer malto-oligosaccharide peaks (15 DPs) were observed in that case over 20 min. Thus, IS can be selected between 150 or 200 mM depending whether a higher number of detected malto-oligosaccharides is required. The variation of MD ladders' signals at lower IS ranges was also verified for the TEOA / Cit BGE (Fig S1 in the ESI). The signals approached the plateau at IS from 100 mM; and the increase in peaks' signals was less significant between 100 - 200 mM. The use of high IS (150 or 200 mM rather than 100 mM or less) is indeed not only for signal improvement, but also for efficient EOF suppression to facilitate the migration of glycans to the detector. IS higher than 200 mM was as well tested but then was not considered for further experiments due to too high currents (more than 65 µA, which can generate pronounced Joule heating for a capillary of 50 µm i.d.). The TEOA/Cit BGE was also compared with the commercial one (SCIEX polymer containing BGE) for successive separations of oligosaccharides (over 15 runs). Peak heights were almost 4-fold higher for TEOA/Cit 200 mM (Fig. 3B) compared to those obtained with the commercial buffer (Fig. 3C). The TEOA/Cit BGE exhibited similar repeatability of migration times (RSD % < 0.8 % over 15 runs for DP 1, DP 6 and DP 10 oligomers, (table S1 in the supplementary material) as with the commercial buffer. Better peak capacity was achieved for TEOA/Cit polymer-free buffers. This advantageous feature can be explained by several features. First, thanks to the drastic reduction of the zeta potential of the inner capillary surface at very high IS of the buffer, significant EOF suppression could be obtained [33, 34]. Second, the efficiency of alkylamines in BGEs for capillary surface coverage via electrostatic interaction with the ionized silanols of the capillary was higher than that offered by monovalent cations (Na^+, Li^+, K^+, NH_4^+) in conventional BGEs [42]. Third, when using very high concentrations of BGE constituents (approaching their saturation), it is assumed that the solvation degree of the silanol groups of the capillary was decreased due to competition with other ions in the electrolytes for hydration. The presence of very concentrated ions in our BGEs, thus, could allow coverage and reduction of the capillary surface charge, decreasing its zeta potential, which in turn gives a similar effect as a ‘virtual coating’ for EOF suppression.

3.1.3. Analysis of human serum glycans from CDG individuals: towards diagnostic applications

For CDG screening via N-glycan analysis, the preferred methods include gel electrophoresis, chromatography and mass spectrometry (MALDI-MS and ESI-MS), whereas CE-LIF has not been listed yet as a well-established method for this purpose [7]. In order to showcase the applicability of our new CE-LIF method for the CDG diagnostics, total N-glycans from two serum samples from CDG patients and one from a healthy person (control) were analyzed by CE-LIF (Fig. 4). Trace (A) corresponds to the control serum, featuring different peaks from biantenated N-glycans (peaks a-b-c). Trace (B) shows the serum glycan profile from a B4GALT1-CDG individual with a quasi-total deficit of galactosylated N-glycans, which differs significantly from the control one. Trace (C) corresponds to a MGAT2-CDG case, where one of the N-glycan antennae presents a major GlcNAcylation defect. An extra peak (peak g at ~11.5 min) that is not present in the control sample was found for this CDG subtype. Accordingly, very typical and distinct profiles are obtained for the different CDG subtypes showing that an adequate diagnosis could be possible with N-glycan profiling by CE-LIF. The peak patterns obtained with our polymer-free BGE were similar to those obtained with the commercial buffer (Fig. S2 in the supporting material), demonstrating the applicability of our approach for the analysis of glycans from biofluids. In both CE-LIF profiles (Fig. 4) and MALDI-MS reference ones [43], featured peaks were found for CDG samples whereas they were non-detectable in the healthy control. It is worthy to note that unlike CE-LIF, no separation step was carried out prior to ionization for MALDI-MS. The MS profiles therefore may present some overlaps of glycans having the same mass to charge ratio and / or presenting poor signals for those hardly ionizable.

3.2. Large volume sample stacking with EOF modulation in uncoated capillaries

3.2.1. Proposed principle

In conventional methods of large volume sample stacking with electroosmotic pumping (LVSEP), which is the most frequently reported approach for electrokinetic preconcentration of glycans, the EOF variation and suppression is established through buffer pH adjustment and the use of neutral capillary coatings [26-29, 37, 44]. Unfortunately, such mode of preconcentration often leads to fast deterioration of the permanent neutral coating layer over runs [37]. Therefore, we took advantage of our new BGE to develop an analyte preconcentration approach without recourse to any capillary coating via large volume sample stacking (LVSS) with EOF modulation (see Fig. 1B). This electrokinetic preconcentration required indeed two completely different EOF magnitudes for sample preconcentration and for the separation of the stacked analytes. High electroosmotic mobility (for example μ_{EO} = $60x10^{-5}$ $cm^2/V/s$ if the sample is prepared in water) was needed to remove the sample matrix from the capillary during the stacking of negatively charged analytes, whereas a much lower one (μ_{EO} ~ 0) was required to ensure the electromigration of enriched glycans to achieve their baseline separation. Here we propose to use variation of the IS of the BGE, rather than the buffer pH (not the pH of the sample matrix) as in conventional approach for modulation and quasi-total reduction of the EOF.

3.2.2. Procedure optimization and performance

The challenging part of this approach was to establish in the same uncoated capillary two distinct EOF ranges that transiently switch from one to another. The very high μ_{EO} (more than 55×10^{-5} $cm^2.V^{-1}.s^{-1}$) could in principle be obtained by using either water or very diluted BGE as the sample matrix to be filled in the capillary before the preconcentration step. Our preliminary optimizations revealed that oligosaccharides prepared in water exhibited the best EOF-assisted stacking performance. Note that if the sample was prepared in deionized water, the very low ion density in the sample matrix could sometimes provoke drop of the current profile during the large volume sample stacking, leading to failure of this preconcentration step. This challenge was overcome (or at least alleviated) by replacing deionized water with tap water to ensure a sufficient ion density in the sample matrix. Then, to reach a very low EOF (μ_{EO} approaching zero) required for CE-LIF separation, extremely concentrated buffers were needed (see section 3.1). In our first attempts, the BGEs having IS less than 100 mM were unable to create a sharp EOF transition for efficient preconcentration-separation of oligosaccharides. Much higher IS (150 mM or higher) were required to maintain the drastic change of μ_{EO} between the two steps.

Finally, the new preconcentration approach was performed with the TEOA/Cit BGE (IS 200 mM). Fig. 5 compares the LVSS-CE-LIF and the CE-LIF method without preconcentration for MD ladders. The monitoring of the current profile during the preconcentration (Fig. S3 in the ESI) showed that the low IS sample matrix filled in the capillary (reflected by the current approaching 0 µA at the beginning) was gradually replaced by the high IS zone of the BGE (as the current increased). The LVSS preconcentration apparently transitioned to the CE-LIF separation mode without operator intervention when the matrix was 100 % removed and the BGE completely filled the capillary (i.e., stable current was reached). Based on the peak heights and injected sample concentrations, a detection sensitivity improvement of 200 times was achieved with the inclusion of LVSS. Besides the contribution of preconcentration effect, this signal enhancement also comes from the fact that the high BGE IS required for EOF elimination during electrophoresis can boost at the same time the sample stacking effect (as shown in Fig. 1A). This resulted in further improvement of peak shapes and detection sensitivity. As the EOF was manipulated through IS changes rather than pH, we could select the best pH conditions for the analytes during preconcentration and separation. The peak sharpness was a bit compromised for the late-migrating peaks in the LVSS-CE-LIF mode. Considering that these peaks resulted from the stacking of a sample volume equivalent to 100 % of the capillary volume (compared to 1 % in the normal CE -LIF mode), this slight change in peak sharpness and resolutions was considered to be tolerable. It is worthy to note that the different degree of polymerization glucose oligomers behaved differently in terms of enrichment factor and resolution changes after LVSS-CE-LIF (see Fig. S4). The improvement of peak height was more pronounced for the smallest DPs, whereas the peak resolution decreased more for them (except for DP1 and DP2) after LVSS process. This was probably due to the different DP responses to the new BGE compositions and/or preconcentration process, as it was already observed in another approach for preconcentration and CE-LIF of MD ladders [21]. The triplicates of electropherograms and current profiles from LVSS-CE of MD ladders (Fig. S5 in the ESI) demonstrated good repeatability of this process, with calculated RSD values less than 5 % for peak areas and 0.3 % for migration times. The current profile was used to monitor the successful completion and repeatability of the LVSS-CE process.

3.2.3. Analysis of N-glycans

To evaluate the performance of the proposed approach with glycoprotein-derived oligosaccharides, the BGE composed of 200 mM TEOA/Cit was used for preconcentration and CE-LIF separation of APTS-labeled N-glycans released from human IgG using PNGase

F. Most of human IgG N-glycans are bi-antennary complex type with low sialylation (5-10 %), highly core-fucosylated (> 92 %); and a small proportion contains a bisecting GlcNAc (11 %) [45]. By referring to the similar peak patterns assigned for human IgG using CE-LIF with the commercial BGE [46, 47], we could assign the corresponding N-glycan structures to the major peaks with our new buffer (Fig. 6A). Other peak identifications could be gleaned from the reference profile provided elsewhere [47]. With the new LVSS-CE-LIF method (Fig. 6B), while the same major peaks were separated with little decrease in resolution, the detection sensitivity was drastically improved (by approximately 160 folds, based on peak height). This in principle would allow more glycans including even minor ones, which are not easily visualized using standard CE-LIF protocol without preconcentration, to be detected. This method can indeed be applied to other negatively charged analytes, with the conditions that their electrophoretic mobilities are smaller than the EO mobility during preconcentration and at the same time higher than the EO mobility during the separation step.

4. Conclusion

In this study we demonstrate the successful development of a new approach for LVSS via EOF modulation without recourse to any capillary coating or polymer addition. EOF can be modulated in a wide range and quasi-totally suppressed just by changing the IS of the BGE. New BGE compositions for CE-LIF were accordingly developed and the best constituents of TEOA/Cit were explored to allow signal improvement (5-fold over conventional protocols) and realization of this new LVSS mode (allowing signal enhancement by 200 times). This preconcentration approach does not require any user's intervention for transition to the subsequent separation step of enriched target molecules. The new mode of LVSS-CE is simple and straightforward and can be implemented on any CE instrument. This opens different alternatives of EOF-assisted preconcentration in fused silica capillaries, which were utilized until now mainly for neutrally coated capillaries. These were successfully applied for separation and detection of glycoprotein-derived N- glycans, potentially offering diagnosis options for glycan-related diseases. Further improvement of glycan signals could be possible in the future by combining the new BGEs with multiple-cycles of large volume sample stacking, which is a recently developed electrokinetic preconcentration strategy [37]. Development of other modes of EOF-assisted preconcentration using these new BGE compositions, as well as their exploitations for sensitive and selective determination / characterization of biomolecules and nanometric entities with CE-LIF are envisaged.

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Table captions:

Table 1. List of BGE compositions at ionic strength of 150 mM and pH of 4.75, as simulated with Phoebus program for a use in a fused silica capillary of 50µm i.d. x 65cm length. The currents were calculated by the Phoebus program for the pre-defined voltage of 30 kV.

Table 2: Comparison of the salient data obtained with different BGEs for CE-LIF of oligosacharides, using uncoated fused silica capillaries (50 µm I.D., 65 cm total length)

Figure captions:

Fig. 1. A) Working principle of the novel BGEs for CE-LIF of oligosaccharides. The ionic strengths (IS) of the BGE composed of large weakly charged molecules are increased to extremely high levels to allow high stacking of target analytes and at the same time suppression of EOF without recourse to any capillary coating. Oligosaccharides can be prepared in water or diluted BGE.

B) Principle of large volume sample stacking with EOF modulation and suppression in an uncoated fused silica capillary used in this study. This mode allows two completely different EOF magnitudes (via variation of the IS of the BGE) for sample preconcentration and for the separation of the stacked analytes.

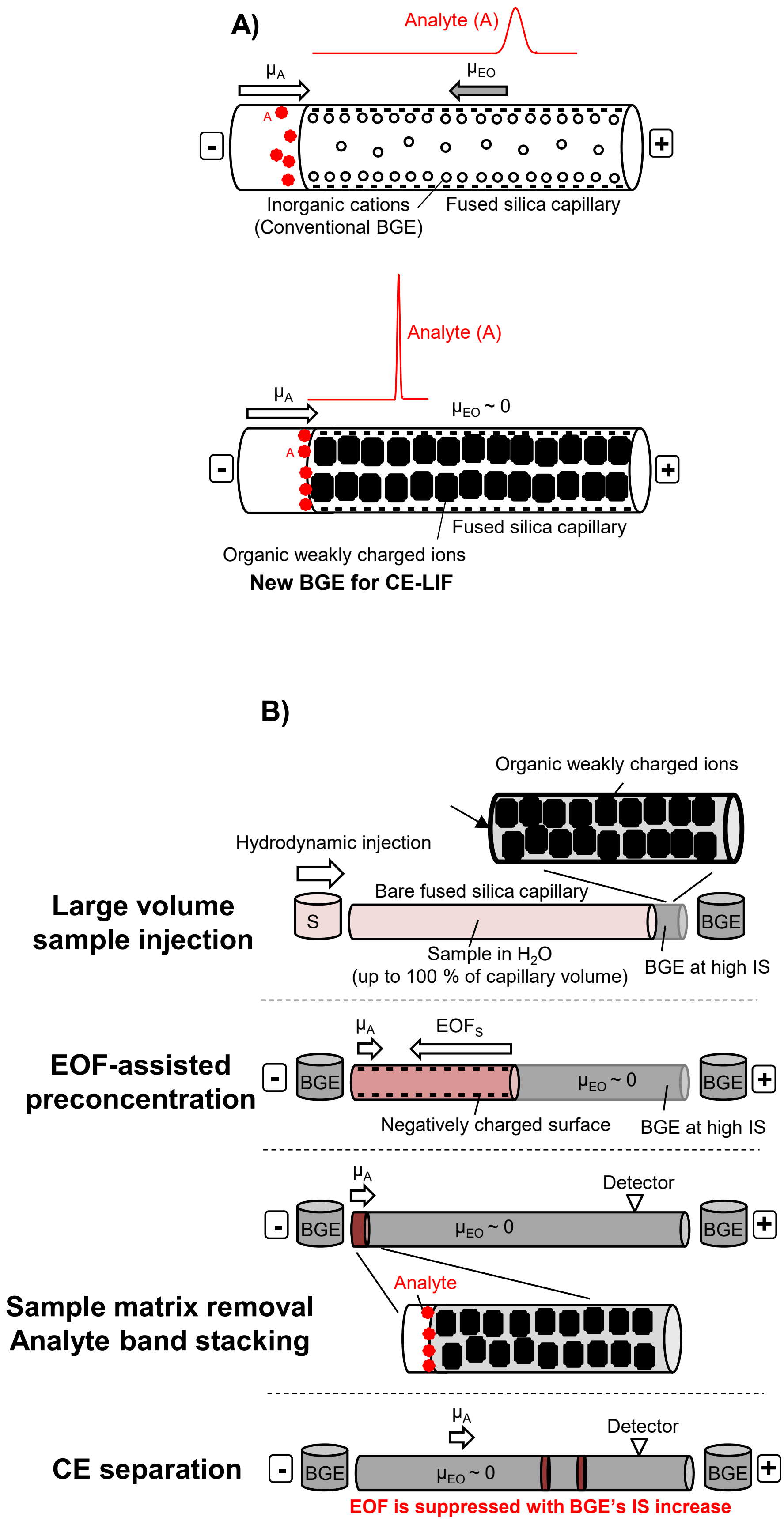
Fig. 2. CE-LIF of oligosaccharides using different BGE compositions at pH 4.75. **A)** LiOH / AcOH IS of 25 mM (LiOH 25mM + AcOH 47 mM); **B)** β-Ala / Trimellitic acid IS of 150 mM (β-Ala 1052 mM and Trimellitic acid 30 mM); **C)** β-Ala / AcOH IS of 150 mM (β-Ala 861 mM and AcOH 127 mM) ; **D)** TEOA / AcOH IS of 150 mM (TEOA 150 mM and AcOH 266 mM); **E)** TEOA / Cit IS of 150 mM (TEOA 150 mM and Cit 58 mM). *F* denotes for residual fluorescent peaks. CE conditions: fused silica capillary, 50 µm i.d., 50/60 cm, hydrodynamic injection at 50 mbar for 10 s, high voltage of -25 kV.

Fig. 3: CE-LIF of oligosaccharides using TEOA/Cit BGE at IS of **A)** 150 mM; **B)** 200 mM; **C)** Commercial Sciex BGE. *F* denotes for residual fluorescent peaks. CE conditions as in Fig. 2

Fig. 4 CE-LIF of N-glycans from human serum of healthy control and CDG individuals, using BGE composed of TEOA / Cit 200 mM in an uncoated silica capillary. A) Control patient, B) B4GALT1-CDG patient and C) MGAT2-CDG patient. Other CE conditions as in Fig. 2.

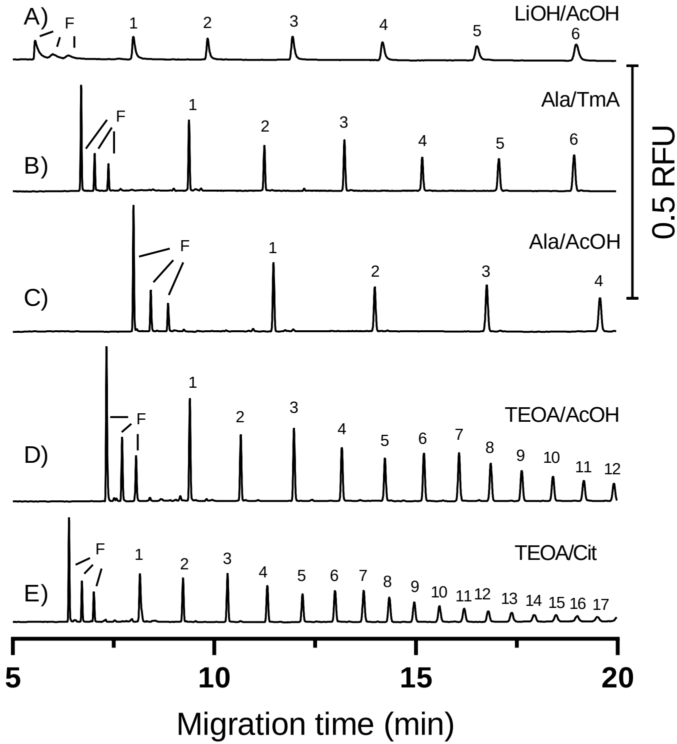
Fig. 5: CE-LIF with and without large volume sample stacking (LVSS) with EOF modulation of oligosaccharides, using BGE composed of TEOA/Cit at IS of 200 mM. **A)** LVSS-CE-LIF of oligosaccharides 200,000x dilution; **B)** CE-LIF of oligosaccharides 2000x dilution. Other conditions as in Fig. 2.

Fig. 6: **A)** CE-LIF of N-glycans released from human IgG; **B)** LVSS-CE-LIF of human IgG glycans using TEOA/Cit BGE at IS of 200 mM. Other CE conditions as in Fig. 2.



A)
Analyte (A)
μA
μEO
A
Inorganic cations
(Conventional BGE)
Fused silica capillary
Analyte (A)
μA
μEO ~ 0
A
Fused silica capillary
Organic weakly charged ions
New BGE for CE-LIF
B)
Organic weakly charged ions
Hydrodynamic injection
Large volume sample injection
S
Bare fused silica capillary
BGE
Sample in H2O
(up to 100 % of capillary volume)
BGE at high IS
EOF-assisted preconcentration
μA
EOFS
BGE
μEO ~ 0
BGE
Negatively charged surface
BGE at high IS
μA
Detector
BGE
μEO ~ 0
BGE
Analyte
Sample matrix removal
Analyte band stacking
CE separation
μA
Detector
BGE
μEO ~ 0
BGE
EOF is suppressed with BGE's IS increase

Figure 1



A)
F
1
2
3
4
5
6
LiOH/AcOH
B)
Ala/TmA
C)
Ala/AcOH
D)
TEOA/AcOH
7
8
9
10
11
12
E)
TEOA/Cit
13
14
15
16
17
0.5 RFU
5
10
15
20
Migration time (min)

Figure 2

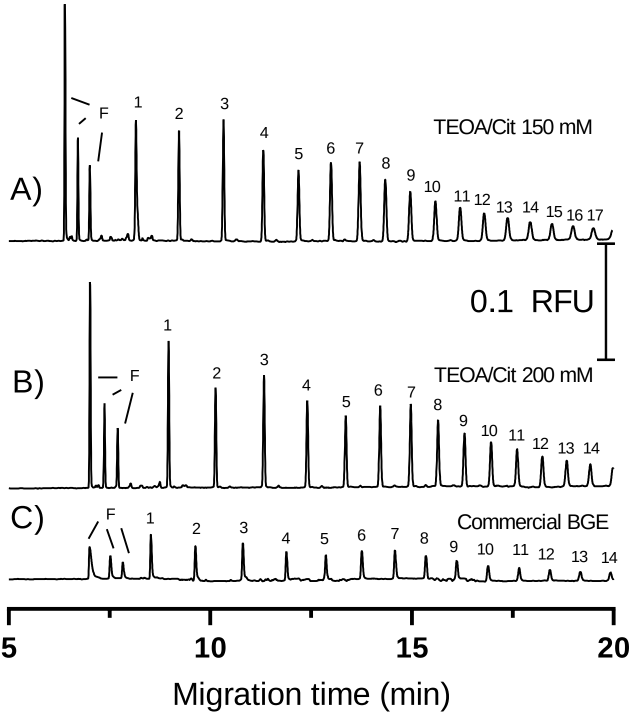
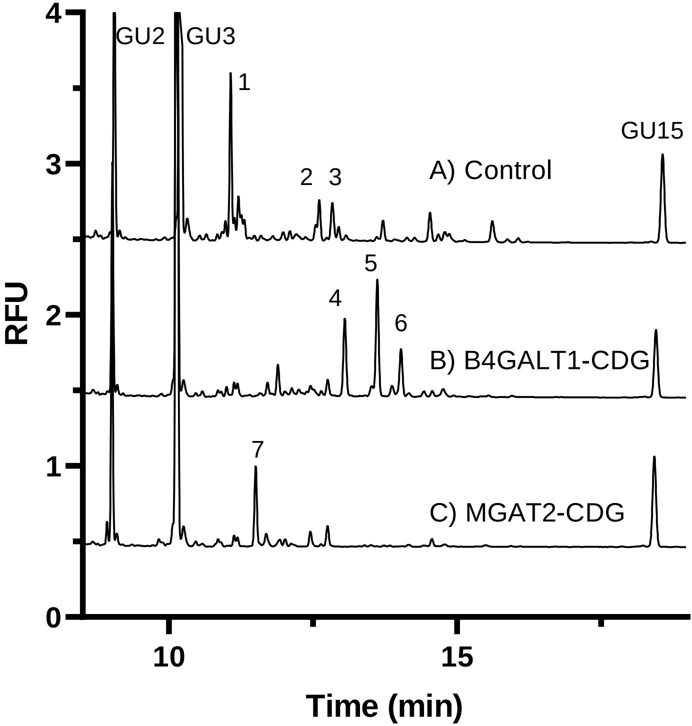

A)
F
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
TEOA/Cit 150 mM
0.1 RFU
B)
F
1
2
3
4
5
6
7
8
9
10
11
12
13
14
TEOA/Cit 200 mM
C)
F
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Commercial BGE
5
10
15
20
Migration time (min)

Figure 3



RFU
4
3
2
1
0
GU2
GU3
1
2
3
GU15
A) Control
5
4
6
B) B4GALT1-CDG
7
C) MGAT2-CDG
10
15
Time (min)

Figure 4

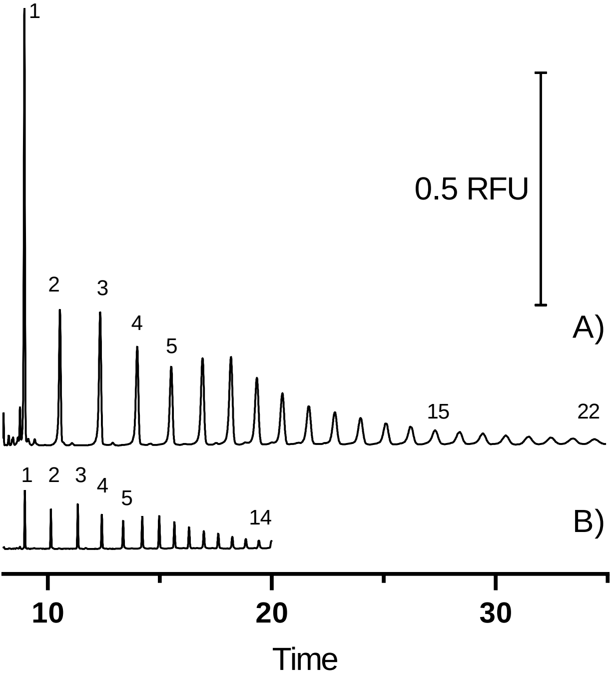


Figure 5

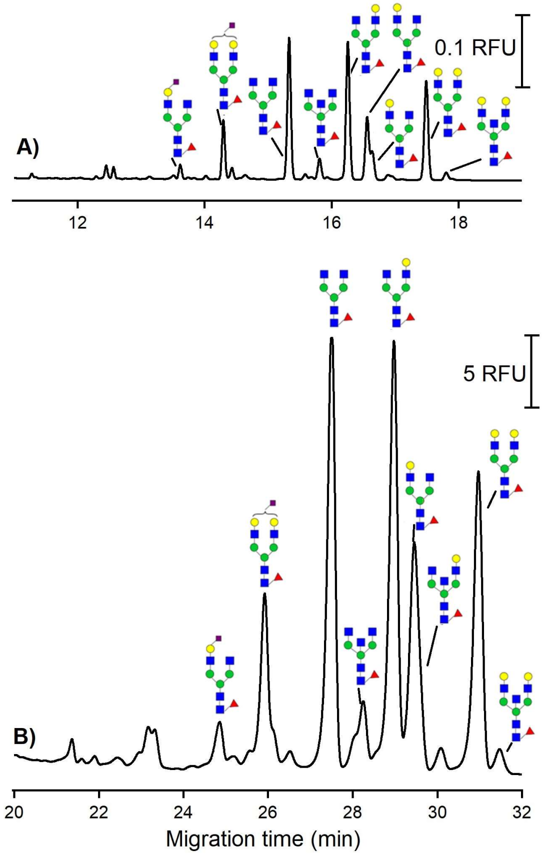


Figure 6

Table 1. List of BGE compositions at ionic strength of 150 mM and pH of 4.75, as simulated with Phoebus program for a use in a fused silica capillary of 50µm i.d. x 60cm length. The currents were calculated by the Phoebus program for the pre-defined voltage of 30 kV.

BGE compositions	**I (µA) at 30kV**	**buffer capacity (mmol/l.pH)**	**Remark**
LiOH 25mM + Acetic acid 47 mM (IS 25 mM)	16	28	Reference BGE
TEOA 150 mM + Acetic acid 266 mM (IS 150 mM)	21	155	Expected good quality
TEOA 150 mM + Citric acid 58 mM (IS 150 mM)	31	44	Expected good quality
β-Ala 1052 mM + Trimellitic acid 30 mM (IS 140 mM)	19	185	Expected good quality
β-Ala 963 mM + Acetic acid 127 mM (IS 70 mM)	57	506	High current generation
β-Ala 861 mM + Pyromellitic Acid 19 mM (IS 150 mM)	16	152	Poor solubility of pyromellitic acid
TEOA 150 mM + Sorbic Acid 270 mM (IS 150 mM)	33	158	poor solubility of sorbic acid
TEOA 150 mM + Gluconic acid 160 mM (IS 150 mM)	27	20	poor solubility of gluconic acid
TEOA 150 mM + aspartic acid 166 mM (IS 150 mM)	31	34	poor solubility of aspartic acid
TEOA 150 mM + anisic acid 210 mM (IS 150 mM)	29	100	poor solubility of anisic acid
TEOA 150 mM + furoic acid 210 mM (IS 150 mM)	31	7	poor solubility of furonic acid

Table 2: Comparison of the salient data obtained with different BGEs for CE-LIF of oligosacharides, using uncoated fused silica capillaries (50 µm i.d., 60 cm total length)

BGE compositions	**Measured current (µA) under HV of -25kV**	**The highest DP detected over a 20 min run**	**Signal / noise ratio for specific DP peaks**		**RSD % for migration time (n = 3)**		**RSD % for peak area (n = 3)**	
			DP1	DP6	DP1	DP6	DP1	DP6
LiOH / AcOH 25 mM	14.5	6	64	48	0.92	0.30	1..37	2.46
β-Ala / Trimel 140 mM	35	6	366	101	0.67	1.42	1.61	2.74
β-Ala / AcOH 70 mM	70	4	414		0.83		0.46	
TEOA / AcOH 150 mM	53	12	646	149	0.28	0.42	1.51	0.59
TEOA / Cit 150 mM	51	17	659	260	0.26	0.21	4.46	1.06

** DP: degree of polymerization of glucose oligomers*