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Sensitive and fast characterization of site-specific protein glycosylation with capillary electrophoresis coupled to mass spectrometry

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ABSTRACT

Glycoproteomic analysis requires efficient separation and sensitive detection to enable the comprehensive characterization of glycan heterogeneity. Here, we report the use of capillary zone electrophoresis-electrospray ionization-mass spectrometry (CZE-ESI-MS) with an electrokinetically-pumped nanospray interface for the study of protein glycosylation microheterogeneity. A fast separation was developed that resolved intact glycopeptides generated from standard proteins within ~9 min. Differentially terminal-galactosylated and sialylated species with the same glycosylation sites were well resolved. The concentration detection limits for CZE were three times higher than for nanoLC methods; however, a 200-fold smaller injection volume was used in CZE, which reflects the use of an extremely efficient electrospray interface in our CZE-ESI-MS setup. The resulting glycopeptide mass detection limit was two orders of magnitude superior to a nanoLC method. We also observed a 1.5% and 7% average relative standard deviation in peak migration time and glycopeptide relative abundance, and a four order of magnitude linear dynamic range in signal intensity. With CZE-ESI-MS, 40 haptoglobin glycopeptides were identified from roughly 40 fmol of digest.

1. Introduction

The characterization of protein glycosylation is important in disease prognosis and in the quality control of recombinant antibodies used as therapeutics [1–5]. This characterization is extremely challenging because the heterogeneity and complexity of glycan is coupled with the challenges of protein analysis. To simplify the analysis, glycans are typically cleaved from the protein, and the two components are analyzed separately. However, this classic approach loses information on the glycan that modifies each site within a protein.

Instead, it is valuable to analyze site-specific protein glycosylation [6–10]. Inevitably, this analysis requires tryptic digestion of the protein, followed by mass spectrometric characterization of the resulting intact glycopeptides. Glycopeptide analysis is challenging, not only because of the increased complexity of both glycans and peptides in mass spectrometry, but also because of the difficulty in resolving glycopeptide species that have wide abundance distributions.

Reversed phase liquid chromatography (RPLC) coupled with electrospray ionization-mass spectrometry (ESI-MS) is the most widely used method for the analysis of site-specific glycosylation heterogeneity [11,12]. This analysis can be improved using a two dimensional (2D) high-low-pH RPLC approach [4], but requires large amounts of sample and long analysis time.

Capillary zone electrophoresis (CZE) has been demonstrated to resolve closely related glycan structures based on their hydrodynamic volume and associated charge [13,14], and is an attractive alternative to conventional RPLC approach for glycopeptide separation. However, the lack of high-sensitivity and highly stable electrospray interfaces has limited the application of CZE in glycoproteomics, especially for site-specific glycosylation heterogeneity analysis [10]. Recently, Kammeijer and colleagues added a dopant enriched nitrogen gas stream to a sheathless interface to improve the sensitivity and repeatability of glycopeptide analysis with CZE-MS [15]. Khatri and colleagues employed a microfluidic CZE system with integrated nanoelectrospray interface for the analysis of intact glycopeptides from alpha-1-acid glycoprotein [10].

Our group has reported three generations of an electrokinetically pumped sheath-flow nanospray CZE-MS interface [16–18]. This sheath-flow interface is robust and provides a stable spray at very low sheath flow rates, which dramatically improves sensitivity. We have used CZE-ESI-MS with this interface for the analysis of sub-picogram amounts an *E. coli* digest [17], and we have identified over 10000 peptides from the HeLa proteome in single runs [19,20].

In this manuscript, we explored the use of our CZE-ESI-MS platform

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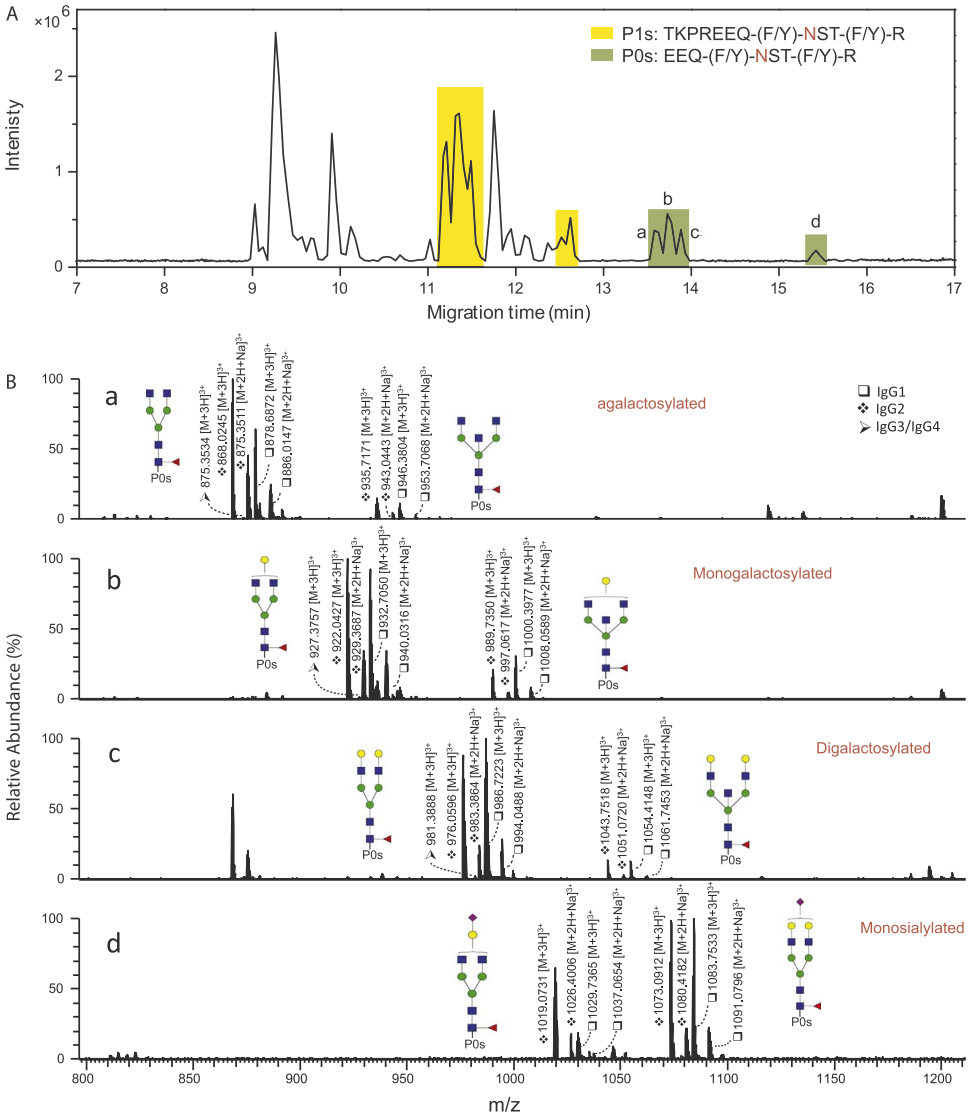


Fig. 1. CZE-ESI-MS analysis of glycopeptides generated from IgG tryptic digest. Glycosylated peptides were enriched with a HILIC column, separated in an 80 cm long, 20/150 μm i.d./o.d. uncoated capillary, and detected with an LTQ Orbitrap Velos mass spectrometer. For this separation, 30 kV was applied at the injection end of capillary and 1.8 kV was applied at the sheath buffer reservoir. A - Base peak electropherograms. B - Summed MS spectra of terminally agalactosylated peptides (a) migrating at 13.53–13.68 min, monogalactosylated peptides (b) migrating at 13.68–13.83 min, digalactosylated peptides (c) migrating at 13.83–13.98 min, and monosialylated peptides (d) migrating at 15.33–15.54 min. Symbols: N-Acetylglucosamine, Mannose, Galactose, Sialic acid, Fucose.

for the separation of intact glycopeptides and the characterization of their site-specific glycan microheterogeneity. A fast separation was developed that resolved glycopeptides generated from a standard protein within ~9 min, yielding two orders of magnitude superior mass detection limit than nanoLC. Asialylated complex glycopeptides were observed to migrate in the order of increasing monosaccharide units; sialylated species with increasing number of sialic acid were separated with higher resolution. Also, our CZE-ESI-MS system produced good reproducibility and wide linear dynamic range.

2. Materials and methods

2.1. Materials and reagents

Bovine pancreas TPCK-treated trypsin, IgG standard from human serum (I4506), haptoglobin standard from human plasma (Hpt, H3536), urea, dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN) and hydrofluoric acid (HF) were purchased from Fisher Scientific (Pittsburgh, PA). Methanol and water were purchased from Honeywell Burdick & Jackson (Wicklow, Ireland).

2.2. Sample preparation

IgG and Hpt (100 μg) dissolved in 100 μL of 50 mM NH_4HCO_3 (pH 8.0) containing 8 M urea (1 μg/μL) were denatured and reduced by the addition of 2 μL of 500 mM DTT at 60 °C for 1 h and then alkylated by the addition of 5 μL of 500 mM IAA at room temperature for 30 min in the dark. After dilution with 900 μL of 50 mM NH_4HCO_3 (pH 8.0) to reduce the urea concentration below 1 M, protein digestion was performed by adding a trypsin solution (1 μg/μL) at an enzyme/substrate ratio of 1/30 (w/w) for 16 h at 37 °C. After acidified with 5 μL of FA, the protein digest was desalted with C18-SepPak column (Waters, Milford, MA), and then lyophilized with a vacuum concentrator (Thermo Fisher Scientific, Marietta, OH).

Glycopeptide enrichment was performed with a locally constructed hydrophilic interaction chromatography (HILIC) spin tip packed with ZIC® glycocapture resin (ProteoExtract® Glycopeptide Enrichment Kit, EMD Millipore, Billerica, MA). 100 μg of IgG and Hpt digests were redissolved in 300 μL of loading buffer (ACN/H_2O/TFA, 80:20:0.1, v/v/v) and then loaded onto the equilibrated tip. After centrifugation at 1500 × *g* for 2 min, the HILIC tip was washed with 400 μL of loading buffer for three times to remove non-specifically adsorbed peptides and then eluted with 200 μL of buffer (ACN/H_2O/TFA, 40:60:0.1, v/v/v). The eluted glycopeptide fraction was collected, divided into two aliquots, and lyophilized using a vacuum concentrator. Each dried

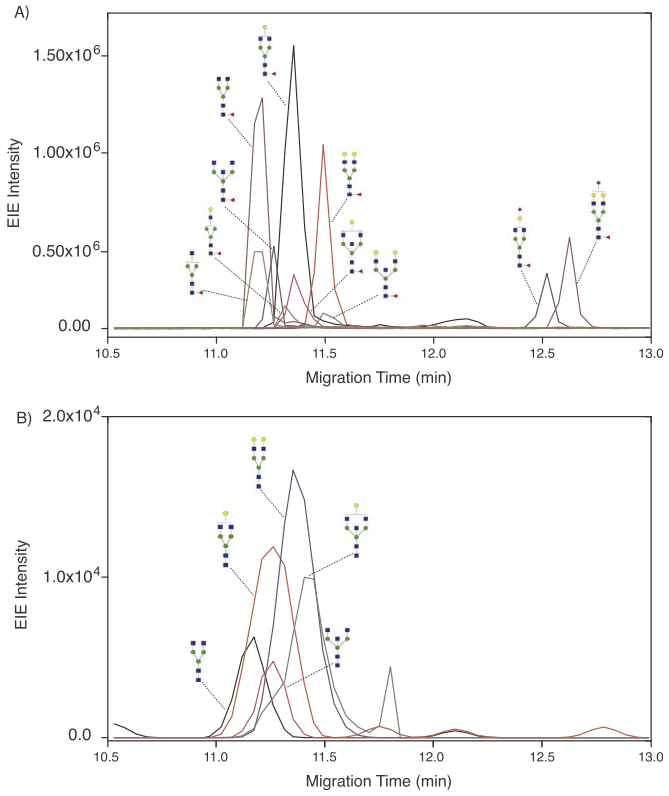


Fig. 2. Extracted ion electropherograms (EIEs) of all (A) fucosylated and (B) non-fucosylated P1 (TKPREEQFN$_{176}$STFR, IgG2) peptides enriched from 34 fmol amounts of IgG digest by CZE-ESI-MS analysis. An Orbitrap Velos mass spectrometer with an 80 cm long, 20/150 μm i.d./o.d. bare capillary was used to generate this data.

glycopeptide fraction aliquot (~1 μg, estimated 2% of overall yield) was stored at −20 °C for further CZE-ESI-MS/MS and LC-ESI-MS/MS analysis.

2.3. CZE-ESI-MS/MS analysis

CZE separation was performed using an uncoated fused silica capillary (20 μm i.d. × 150 μm o.d. × 80 cm length, Polymicro Technologies, Phoenix, AZ). The injection end of the capillary was fixed in a block that allowed pumping fluids with either pressure or voltage [21]. The background electrolyte for the separation was 0.5% (v/v) FA. The capillary was preconditioned by sequentially washing with 1 M NaOH, water, and background electrolyte at 30 psi for 15 min.

CZE was coupled to ESI-MS via a third generation electrokinetically pumped sheath-flow nanospray interface, Fig. S1 [18]. The electrospray interface was constructed from a plastic cross. The distal end of the separation capillary was etched using hydrofluoric acid to reduce the o.d. to ~50 μm [17]. **Caution: use appropriate safety procedures while handling HF solutions.** An emitter was prepared from a borosilicate glass tube (0.75 mm i.d. × 1.0 mm o.d. × 10 cm length). The tubing was pulled to a 25–27 μm outer diameter tip using a P-1000 flaming/brown micropipet puller (Sutter Instruments, Novato, CA). The emitter was placed in one arm of the cross. The separation capillary was threaded through the cross into the emitter. The other two arms of the cross were connected to a sheath buffer reservoir with plastic tubing and to a syringe filled with sheath electrolyte for system flushing. The sheath electrolyte was 10% (v/v) methanol and 0.1% (v/v) FA.

An LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) was operated in positive ion mode. The temperature of the ion transfer capillary was 300 °C. A full scan MS acquired from m/z 350 to 2000 was followed by five data dependent MS/MS events on the five most intense ions. One microscan was set for each MS and MS/MS scan.

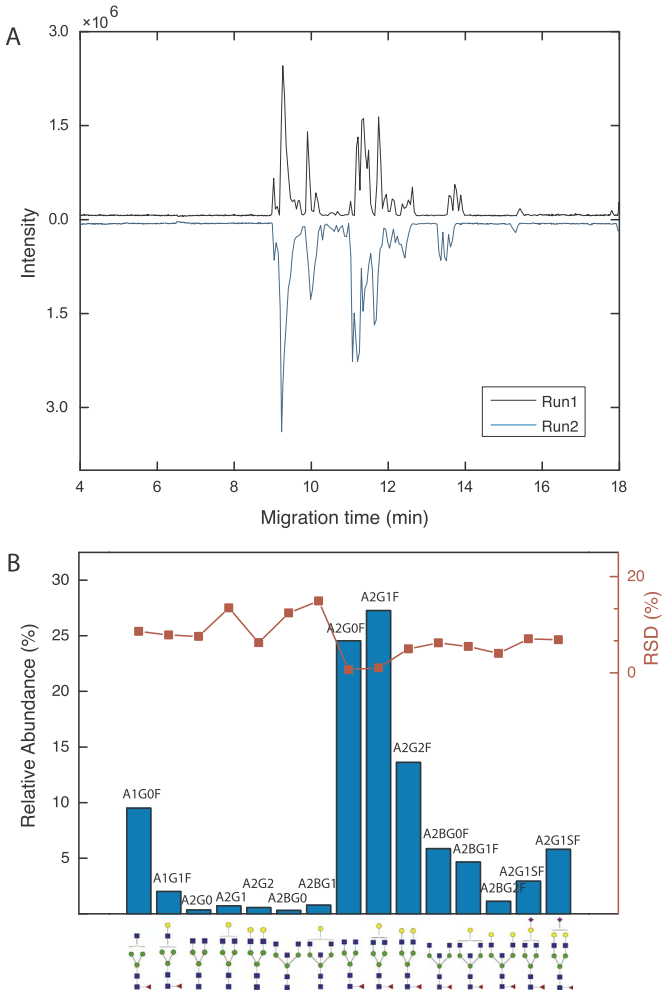


Fig. 3. Reproducibility of CZE-ESI-MS analysis of IgG digest after enrichment on a HILIC column. A – butterfly plot of duplicate runs. B - relative abundance of the glycoforms contributing to the N$_{176}$ glycosylation site of IgG2 and the corresponding RSD value in triplicate runs on the same CZE-ESI-MS setup using an LTQ Orbitrap Velos mass spectrometer.

The mass resolution was set at 30,000 for full MS. The dynamic exclusion function was set as follows: repeat count, 1; repeat duration, 20 s; exclusion duration, 30 s. The target value was 1.00 × 10^6, and the maximum injection time was 500 ms. Higher energy collision dissociation (HCD) was performed at normalized collision energy of 40%, and the activation time was set as 0.1 ms.

The dried glycopeptide aliquots (from 50 μg of initial digest) were redissolved in 5 μL of an electrolyte consisting of 30% (v/v) ACN and 0.05% (v/v) FA, followed by serial dilution by 10, 100, 1000, and 5000 times with the same electrolyte. Samples were injected hydrodynamically for 5 s at 30 psi, producing an injection volume of ~5 nL. Each sample was analyzed in triplicate. During separation, 30 kV was applied at the injection end of capillary and 1.8 kV was applied at the sheath buffer reservoir for electrospray using a Spellman CZE 1000 R high-voltage power supply.

2.4. High-speed CZE-ESI-MS/MS analysis

The fast glycopeptide separation was carried out using an uncoated fused silica capillary (20 μm i.d. × 150 μm o.d. × 42 cm length). The sample was loaded by pressure at 30 psi for 1 s, producing an injection volume of ~2 nL. Other electrophoresis conditions were the same as those employed in the conventional separation.

A Q Exactive HF mass spectrometer (Thermo Fisher Scientific) was

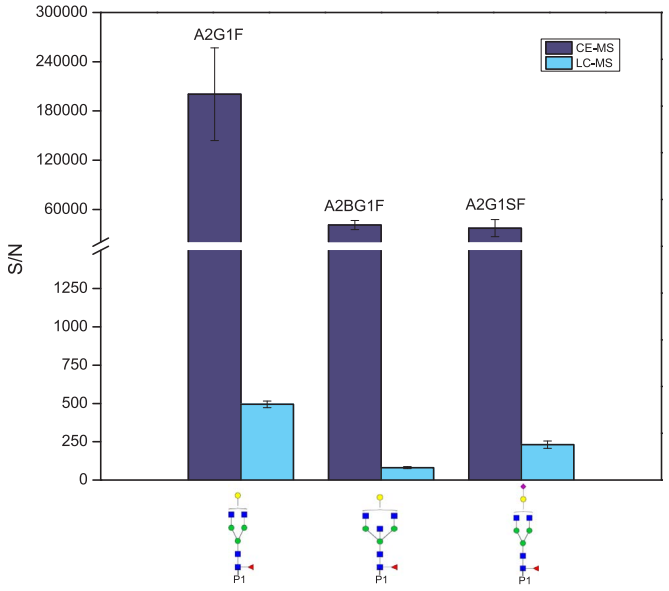


Fig. 4. S/N ratio of IgG2 glycopeptides in CZE-ESI-MS versus nanoLC-ESI-MS analysis. The same LTQ Orbitrap Velos mass spectrometer was employed in both setups. Assigned glycoforms are presented with TKPREEQFN$_{176}$STFR backbone. Equal injection amounts of analyte were used for CZE and nanoLC analysis. Errorbars are standard deviations (n = 3).

used for the fast glycopeptide analysis. The temperature of the ion transfer capillary was 300 °C. A top-five data dependent acquisition method was used. Full MS scans were acquired in the Orbitrap mass analyzer over *m/z* 350–2000 with the resolution of 60,000. The target value was 3.00 × 10^6 and the maximum injection time was 50 ms. The dynamic exclusion duration was 30 s. HCD was performed at normalized collision energy of 28% and the activation time was set as 0.1 ms. The resolution of the MS/MS scan was set at 35000. For Hpt glycopeptides analysis, two scan ranges were used. For most studies, an *m/z* range from 350 to 2000 was used. To identify low abundant glycopeptides, an *m/z* range from 1350 to 1850 was used.

2.5. UPLC-ESI-MS/MS analysis

Glycopeptides (IgG) were analyzed by a nanoRPLC-ESI-MS/MS system, consisting of a nanoACQUITY UltraPerformance LC® (UPLC®) system (Waters, Milford, MA, USA) and an LTQ Orbitrap Velos mass spectrometer. Separation was performed using a commercial C18 reversed phase column (Waters, 100 µm ×100 mm, 1.7 µm particle, BEH130 C18, column temperature 40 °C). Injection volume was 1.0 µL. An emitter (Silica TipTM, New Objective, Woburn, MA) with a 20 µm inner diameter and 10 µm tip was employed for nanospray. Solvent A (0.1% FA in H_2O) and B (0.1% FA in ACN) were used to establish a 30 min gradient, comprised of 10 min of 2% B, then 1 min of 2–8% B, 16 min of 8–28%, 1 min of 28–90% B, and finally maintained at 90% B for 2 min, with the flow rate at 0.8 µL/min. The column was equilibrated for 10 min with 2% B at 0.8 µL/min before the analysis of next sample. The LTQ Orbitrap Velos mass spectrometer was operated in positive mode with a 1.8 kV applied spray voltage. Other operating parameters were the same as the CZE-ESI-MS/MS section.

Hpt glycopeptides were analyzed by a nanoRPLC-ESI-MS/MS system constructed with Q Exactive HF mass spectrometer for comparison with the high-speed CZE-ESI-MS protocol. A 80 min long gradient (14 min of 2% B, 1 min of 2–8% B, 60 min of 8–35%, 1 min of 28–90% B, and 90% B for 5 min) was used for the separation. Other chromatographic conditions were the same as the IgG analysis. The Q Exactive HF instrument was operated in positive mode with a 1.9 kV applied spray voltage. Other operating parameters were the same as the high-speed CZE-ESI-MS/MS section.

2.6. Data analysis

CZE-ESI-MS/MS and UPLC-ESI-MS/MS data were analyzed with Xcalibur software (Thermo Fisher Scientific). MS and MS/MS data were used to manually assign the intact glycopeptide structure. Extracted ion electropherograms and extracted ion chromatograms were created using a 10 ppm mass tolerance window for theoretical masses corresponding to the target glycopeptides.

3. Results and discussion

The goal of this work is to evaluate CZE-ESI-MS as a tool for the determination of site-specific glycan microheterogeneity. Two glycoproteins were analyzed under conventional and high-speed protocols.

3.1. Glycopeptide migration in CZE

HILIC solid-phase extraction was used to isolate glycopeptides from an IgG standard digest. Glycopeptides generated from roughly 34 fmol of the IgG digest were loaded onto an 80 cm long uncoated fused silica capillary and electrophoretically separated. Two peptides whose sequences differ based on missed cleavages were detected [4], Fig. 1. These sequences are named P0 and P1, and each peptide generates a number of different glycosylation patterns. Based on the differences in the peptide sequence of each isotype included in the polyclonal IgG standard, P0 peptides were classified as EEQYN$_{180}$STYR (IgG1), EEQFN$_{176}$STFR (IgG2), and EEQYN$_{227}$STFR/EEQFN$_{177}$STYR (IgG3/IgG4) [5]. These glycopeptides are noted in the base peak electropherogram, Fig. 1A, where the P1 group (yellow) migrated at 11.2–11.7 and 12.4–12.8 min, while the P0 group (green) migrated from 13.5 to 15.5 min. The separation was complete in 16 min.

Glycopeptides with the same peptide sequence but different glycan

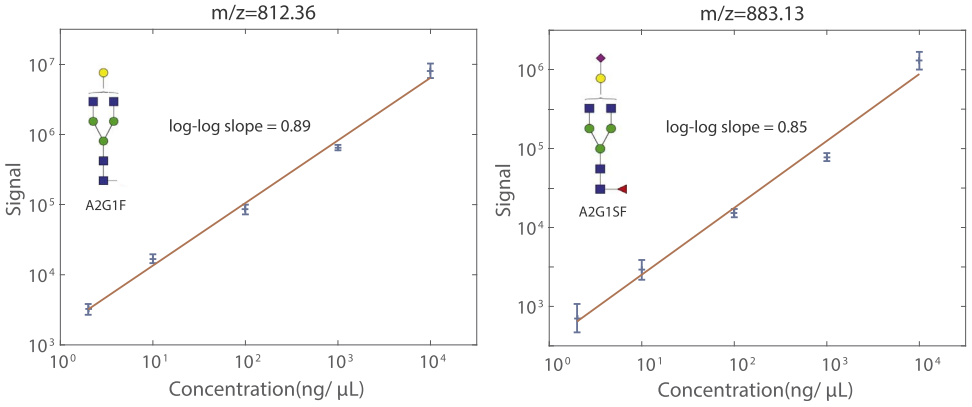


Fig. 5. Linearity of the CZE-ESI-MS method. The extracted ion electropherogram intensity of A2G1F glycopeptide (A) and A2G1SF glycopeptide (B) with the increased amount of loading sample (68–340,000 amol of initial IgG digest). Assigned glycoforms are presented with TKPREEQFN$_{176}$STFR backbone. Error bars are standard deviations (n = 3).

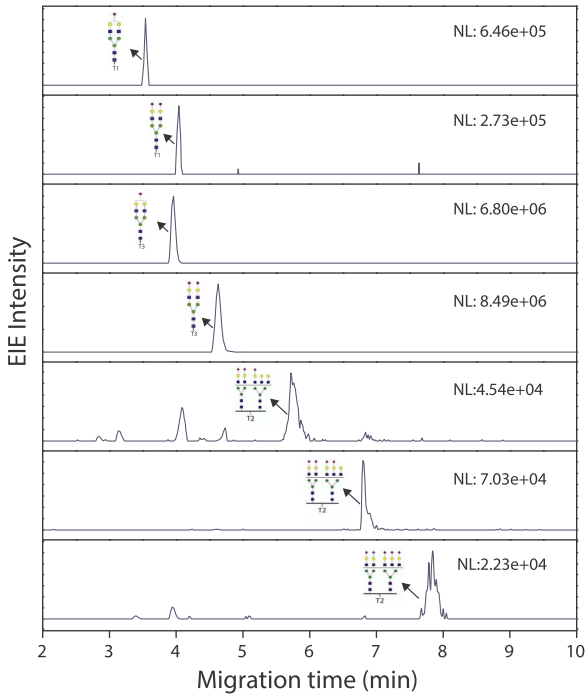


Fig. 6. Extracted ion electropherograms (EIEs) of the most abundant T1, T3, and differential sialylated T2 glycosylated peptides, enriched from haptoglobin. A Q Exactive HF mass spectrometer with a 42 cm long, 20/150 μm i.d./o.d. uncoated capillary was used for CZE-ESI-MS/MS analysis. For this separation, 30 kV was applied at the injection end of capillary and 1.8 kV was applied at the sheath buffer reservoir.

structures were resolved by CZE. Typically, complex biantennary glycans with variable core fucose, bisecting N-acetyl-D-glucosamine (GlcNAc), and terminal galactose and sialic acid contribute to the glycan heterogeneity at the single N-glycosylation site of human IgG [22]. Fig. 1B presents the sum MS spectrum of each P0 peak (a, b, c, and d) shown in Fig. 1A. Asialylated (peak a, b, and c) and monosialylated (peak d) glycopeptides were well resolved. As expected, the increased negative charge and hydrodynamic volume of sialylated species resulted in slower migration. Among the neutral species, migration occurred in the order of increasing number of galactose residues. Almost baseline separation was achieved between terminally agalactosylated (peak a), mono- (peak b), and digalactosylated (peak c) peptides with peak width of about 9 s.

Similar resolution was observed for differentially terminal-galactosylated and sialylated P1 species (Fig. 2). The contribution of a bisecting GlcNAc to the increased migration time of associated glycopeptides was observed on nonfucosylated P1 peptides (Fig. 2B). The resolution of neutral species appeared to be depended on the number of monosaccharide units and the corresponding hydrodynamic volume. Glycoforms identified from IgG standard in this run are listed in Table S1. For comparison, extracted ion chromatograms of high-abundance P1 glycopeptides obtained by nanoRPLC-ESI-MS analysis are shown in Fig. S2. We observe that RPLC separated the glycopeptides based on differences in glycan hydrophilicity and sialic acid number, as has been reported earlier [4,23]; however, the resolution between glycopeptides with the same peptide sequence was much lower than that obtained by CZE, especially for differently sialylated species.

We also evaluated the CZE-ESI-MS system coupled with a linear polyacrylamide coated capillary (30 μm i.d. × 150 μm o.d. × 80 cm) for glycopeptide separation (Fig. S3). The linear polyacrylamide coating produces low electroosmosis and relatively long separations, which are ideally suited for very deep single-shot proteomic analysis [19]. Although resolution improved for peptides modified with neutral and sialylated glycans compared to the uncoated capillary (Fig. 2A), relatively modest improvements were observed for the resolution of terminally agalactosylated, mono-, and digalactosylated peptides.

3.2. Repeatability

The repeatability of the system was investigated by loading glycopeptides generated from roughly 34 fmol of the IgG digest. The sample was separated and detected by CZE-ESI-MS with an LTQ Orbitrap Velos mass spectrometer, Fig. 3A. Peak profiles and intensities are reasonably reproducible between runs. On the basis of triplicate analysis, the average RSD of the relative abundance of identified IgG2 glycopeptides with the TKPREEQFN$_{176}$STFR backbone (P1) was 7%. Higher RSDs were observed for A2G1 (13%), A2BG0 (12%), and A2BG1 (15%) glycopeptides, all of which had very low relative abundance (0.7%, 0.3% and 0.8%, respectively, Fig. 3B). The summary of identified glycopeptides is provided in Table S1.

3.3. Limits of detection, linearity, and carry-over

The detection limit was estimated for intact glycopeptides and compared with that obtained by a conventional nanoRPLC-ESI-MS system. We first manually extracted electropherograms for three IgG2 glycopeptides with different abundance distributions (neutral A2G1F, bisected A2BG1F, and sialylated A2G1SF) from the separations generated by loading the HILIC fraction enriched from 51 ng of IgG digest. As shown in Fig. 4, CZE-ESI-MS generated a two-order of magnitude higher signal-to-noise ratio than nanoRPLC-ESI-MS, with the same LTQ Orbitrap Velos mass spectrometer and with the same injected sample amount of glycopeptides.

A calibration curve was generated by analysis of a serial dilution of A2G1F and A2G1SF glycopeptides of IgG2. The signals for the glycopeptides increased linearly with loading amount across four orders of magnitude, Fig. 5. When the glycopeptides were enriched from 10 pg of IgG digest, the A2G1F glycopeptide generated signal-to-noise ratio (S/N) of 30 ± 3. Assuming that IgG2 formed 50% of the total protein amount based on the MS signals from IgG1 and IgG2 peptides, and based on its molecular weight (150 kDa), ~30 amol of glycopeptides from IgG2 were taken for analysis. The concentration detection limits for CZE were three times higher than for nano-LC, despite the 200-fold smaller injection volume used in CZE. The mass detection limit for CZE (S/N = 3) was ~3 amol, which is a 300-times improvement compared with the 1 fmol LOD obtained by nanoLC-ESI-MS analysis. The performance of CZE reflects the outstanding ionization efficiency of the electrospray interface.

We also evaluated the carry-over of CZE-ESI-MS by analyzing a blank sample immediately following the highest calibration standard, and a lowest calibration standard right immediately following the blank. As shown in Fig. S4, no peak was observed for the extracted glycopeptide in the blank injection, demonstrating the very low sample carry-over produced by CZE-ESI-MS.

3.4. High-speed analysis

To speed the separation, we employed a shorter, uncoated fused capillary (20 μm i.d. × 150 μm o.d. × 42 cm) coupled to a Q-Exactive HF mass spectrometer. The separation of glycopeptides generated from 13 fmol of IgG injected onto the column was performed at 30 kV, corresponding to an electric field > 700 V/cm. The total analysis time decreased to 5.5 min, Fig. S5. Glycopeptide clusters based on the P0 and P1 peptide sequences were well resolved. Sialylated and neutral glycopeptides were nearly baseline resolved, and glycopeptides with zero, one, and two terminal galactoses were partially resolved.

We applied this high-speed CZE-ESI-MS protocol for the analysis of Hpt, which has four N-linked glycosylation sites (N_{184}, N_{207}, N_{211}, and N_{241}) on its β chain [6]. Glycopeptides generated from roughly 40 fmol of the Hpt digest were injected. Conventional tryptic digestion of Hpt generates three glycopeptide clusters (T1, T2, and T3, with T2 having

two glycosylation sites). As shown in Fig. 6, T1 (MVSHHN1_{84}LTTGATLINEQWLLTTAK) and T3 (VVLHPN$_{241}$YSQVDIGLIK) glycopeptides, which are more hydrophobic and tend to be more strongly retained on C18 stationary phase, migrated first at ~5 min. Differentiately sialylated species were well resolved. The dramatically increased glycan size and negative charge on sialic acids decreases the electrophoretic mobility of the hydrophilic diglycosylated T2 (N_{207}LFLN$_{221}$HSENATAK) glycopeptides, resulting in their later migration in the electropherogram; these peptides nevertheless migrated in under 9 min. With this high-speed CZE-ESI-MS approach, we achieved the detection of 40 intact glycopeptides from hpt, compared with 35 glycopeptides by nanoRPLC-ESI-MS (Table S2 and S3), which required more sample (1 μg v.s. 2 ng of initial digest) and longer analysis time (80 min v.s. 9 min).

4. Concluding remarks

We describe a CZE-ESI-MS system using an electrokinetically-pumped nanoelectrospray interface. This system provides fast separation, high sensitivity, good reproducibility, and wide linear dynamic range for the analysis of intact glycopeptides. This study demonstrates the potential for CZE-ESI-MS to characterize the site-specific glycan microheterogeneity of proteins, especially for mass-limited samples. Considering the different separation behavior of CZE and RPLC for both the peptide backbone and glycan moiety of intact glycopeptides, CZE-ESI-MS provides a complementary separation to conventional RPLC-ESI-MS/MS in larger scale site-specific glycosylation analyses.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2017.10.015.

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