Unravelling the sugar-coating of prostate-specific antigen : method development and its application to prostate cancer research

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DOPANT ENRICHED NITROGEN GAS

COMBINED WITH SHEATHLESS CAPILLARY ELECTROPHORESIS - ELECTROSPRAY IONIZATION - MASS SPECTROMETRY FOR IMPROVED SENSITVITY AND REPEATABILITY IN GLYCOPEPTIDE ANALYSIS

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ABSTRACT

Over the last years, numerous strategies have been proposed to enhance both ionization efficiency and spray stability in electrospray ionization (ESI), in particular for nanospray applications. In nano-liquid chromatography - mass spectrometry (nano-LC-ESI-MS), a better ESI performance has been observed when a coaxial gas flow is added around the ESI emitter. Moreover, enrichment of the gas with an organic dopant has led to an improved desolvation and ionization efficiency with an overall enhanced sensitivity. In this study, the use of a dopant enriched nitrogen gas (DEN-gas) combined with sheathless capillary electrophoresis (CE)-ESI-MS was evaluated for glycopeptide analysis. Using acetonitrile as a dopant, an increased sensitivity was observed compared to conventional sheathless CE-ESI-MS. Up to 25-fold higher sensitivities for model glycopeptides were obtained, allowing for limits of detection unachieved by state-of-the-art nano-LC-ESI-MS. The effect of DEN-gas on the repeatability and intermediate precision was also investigated. When compared to previously reported nano-LC-ESI-MS measurements, similar values were found for CE-ESI-MS with DEN-gas. The enhanced repeatability fosters the use of DEN-gas sheathless CE-ESI-MS in protein glycosylation analysis, where precision is essential. The use of DEN-gas opens new avenues for highly sensitive sheathless CE-ESI-MS approaches in glycoproteomics research, by significantly improving sensitivity and precision.

INTRODUCTION

Electrospray ionization (ESI) - mass spectrometry (MS) hyphenated to chromatographic and electrophoretic separation techniques is a key technology in proteomics and glycoproteomics. Glycosylation is known to influence protein structure and function in multiple manners. An important aspect is the efficient and stable formation of gas-phase ions from solution. In nano-liquid chromatography (LC)-ESI-MS, the flow rates are typically kept in a range of 10 - 1000 nL/min and used in combination with a spray emitter with a narrow internal diameter, leading to the formation of sub-µm diameter droplets.[185,186] Placing the emmiter tip close to the MS entrance results in an improved sampling efficiency towards the MS without the need for pneumatic assistance. However, the early limitations of nanospray ionization, *e.g.,* poor spray stability and overall low robustness, have driven the development of more adapted emitter geometries (with respect to inner and outer diameter and aperture of the needle) and configurations which allow for a better desolvation and ion transmission.[187-190]

Both droplet desolvation and sampling efficiency may be further improved by adding solvent vapors to the source,[191] a strategy that recently has been made commercially available by Bruker Daltonics.[132] Used with a patented closed ionization source,[192] the vapor-enriched gas flows coaxially around the ESI emitter. In combination with nano-LC-MS and by using acetonitrile (MeCN) as dopant, this commercial setup has shown to increase the ionization efficiency of peptides with a factor of *ca.* 2.6-fold.[193,194] This setup, hereafter referred to as dopant enriched nitrogen (DEN)-gas, has also shown its benefits in glycopeptide analysis by providing a more than 10-fold improved sensitivity since glycosylated species usually show lower ionization efficiencies compared to non-glycosylated species.[195-197]

Capillary electrophoresis (CE) is a miniaturized technique working in the nano-flow regime leading to high sensitivities especially when used with a sheathless interface. The commercial sheathless CE-ESI-MS setup (CESI technology, SCIEX, Framingham, MA, US) is based on a sheathless interface designed by Moini where the porous end of the separation capillary which is inserted into the grounded needle and filled with conductive liquid, providing electrical contact via the pores.[120] This interface has shown to display a mass-flux sensitive response at CE-ESI flow rates lower than *ca.* 25 nL/min, with maximum intensity obtained at very low flow rates (< 10 nL/min).[112] Applications in proteomics and glycoproteomics emphasized the higher sensitivities that can be obtained with such a setup compared to nano-LC-ESI-MS, especially when used in combination with on-line pre-concentration techniques such as transient isotachophoresis (t-ITP).[174,177,198-200]

In the present study, the DEN-gas supply was connected to the commercial sheathless CE-ESI-MS system via an in-house built cone. The performance of this setup was evaluated for

glycopeptide analysis. ESI and MS parameters were optimized with tryptic glycopeptides from polyclonal immunoglobulin G subclass 1 (IgG1). MeCN, isopropanol (IPA) and ethanol (EtOH) were investigated as dopants, as well as different injection volumes. The effect of the flow rate on MS signal was also systematically evaluated. The DEN-gas setup was compared to a conventional sheathless CE-ESI-MS platform without DEN-gas and a state-of-the-art nano-LC-ESI-MS platform with DEN-gas, highlighting relevant differences in terms of limits of detection (LODs) and charge state distribution. Finally, the influence of the DEN-gas setup on glycopeptide analysis of two other model glycoproteins was studied.

MATERIALS AND METHODS

CHEMICALS, REAGENTS AND ENZYMES

IPA, EtOH and methanol (MeOH), all of LC-MS grade, as well as ammonium bicarbonate and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany). Glacial acetic acid (HAc), hydrochloric acid (HCl), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium acetate (AAC), formic acid (FA), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Steinheim, Germany) and were of analytical grade or higher. Porcine trypsin was obtained from Promega (Madison, WI) and TPCK treated trypsin from bovine pancreas was obtained form (Sigma-Aldrich). Milli-Q water (MQ) was obtained from a Q-Gard 2 system (Merck Millipore, Amsterdam, Netherlands). MeCN of LC-MS grade originated from Biosolve (Valkenswaard, the Netherlands).

SAMPLES

The purified native (polyclonal) IgG and IgG1 standards from human plasma were obtained from Athens Research and Technology (Athens, GA). IgG recombinant human monoclonal antibody was produced in CHO cells (IgGmAb1). Polyclonal IgG1 standard was used for the determination of optimal CE-ESI-MS operating conditions and dopant choice after tryptic cleavage and glycan enrichment (see **S-3-1-1** and **S-3-1-2**, **Supporting Information**). A more complex standard was used for the investigation of the injection volume, namely tryptic polyclonal IgG peptides (including the different subclasses, prepared without hydrophilic interaction liquid chromatography (HILIC) enrichment). In-house data was available of IgGmAb1 (monoclonal) for nano-LC-ESI-MS and HILIC-fluorescence (FLD) platforms and IgGmAb1 after tryptic cleavage was therefore used for all other experiments with the CE-ESI-MS allowing comparison between the different platforms. Prostate-specific antigen (PSA) originated from human seminal fluid and haptoglobin (Hp) from human serum, both acquired from Lee BioSolutions (St. Louis, MO). The tryptic cleavage procedures used for the proteins are described in **S-3-1-1**, **Supporting Information**.

CAPILLARY ELECTROPHORESIS - ELECTROSPRAY IONIZATION - MASS SPECTROMETRY

CE experiments were carried out on a SCIEX/Beckman Coulter CESI 8000 system (SCIEX, Framingham, MA) equipped with a temperature-controlled sample tray and a power supply able to deliver up to 30 kV. Separation was performed using bare fused silica capillaries (90 cm × 30 µm i.d. × 150 µm o.d.) and carried out by applying a voltage of 20 kV without any pressure applied. Prior to each sample injection, the capillary was rinsed with 0.1 M NaOH for 2.5 min, 0.1 M HCl for 2.5 min, water for 4 min, and background electrolyte (BGE) for 4 min. The BGE was composed of a solution of 10% AA, *v/v* (pH 2.3). Prior to injection, samples were dissolved in 250 mM AAC at pH 4.0 (3:2, *v/v*) which acted as the leading electrolyte for the on-line pre-concentration step, namely t-ITP.

All samples were injected using hydrodynamic injection with an application of 5 psi for 60 s (corresponding to 6.9% of the capillary volume, 44 nL injected), except PSA that was injected by applying a pressure of 1 psi for 60 s (corresponding to 1.4% of the capillary volume, 9 nL injected). The performance of different injection volumes was evaluated by application of different pressures between 1 and 40 psi for 60 s (corresponding to 1.4 - 55.0% of the capillary volume, 9 - 350 nL injected). In all experiments, a post-plug of BGE followed sample injection by applying a pressure of 0.5 psi for 25 s (corresponding to 0.3% of the capillary volume). The dependence of signal intensity on the flow rate was evaluated in triplicate with and without DEN-gas via direct infusion of purified IgGmAb1 in 10% HAc (0.17 ng/nL) at flow rates in the range 1 - 88 nL/min.

The CE apparatus was hyphenated to a UHR-QqTOF maXis Impact HD mass spectrometer from Bruker Daltonics (Bremen, Germany) via a sheathless CESI-MS interface based on a custom made platform from SCIEX/Beckman Coulter (Brea, CA) allowing for an optimal position of the capillary porous tip in front of the MS nanospray shield (Bruker Daltonics). For experiments with DEN-gas, an in-house made polymer cone was slid onto the housing of the porous tip as illustrated in **Figure S-3.1**, **Supporting Information**, allowing for a coaxial sheath flow of the DEN-gas around the ESI emitter. The concentration of MeCN in the DEN-gas has been experimentally determined and corresponds to *ca.* 4% (mole percentage). All CE-ESI-MS experiments were carried out in ESI positive mode with a capillary voltage of 1200 V. MS parameters including drying gas flow rate and temperature as well as quadrupole ion energy and collision cell energy were similar for both setups. Optimal values are listed in **Table S-3.1**, **Supporting Information**. MS data was acquired between *m/z* 200 and 2000 with a spectral acquisition rate of 1 Hz.

DATA ANALYSIS

CE-ESI-MS and nano-LC-ESI-MS data was analyzed with DataAnalysis 4.2 (Build 387, Bruker Daltonics), except for the direct infusion experiment. MS spectra were recalibrated using sodium adducts detected at the beginning of the electrophoretic run. Extracted ion electropherograms (EIEs, smoothed with a Gaussian fit) were generated for the first three isotopes of the doubly, triply and quaternary charged analytes with a width of ± 0.05 *m/z* units. For PSA and Hp glycopeptides, fragmentation spectra were acquired for 68% of the compositions (**Figures S-3.2 - S-3.7**, **Supporting Information**). IgG and IgGmAb1 glycopeptides were assigned according to literature.[201] All compositions were matched based on exact mass, selectivity and relative intensities (**Tables S-3.2 - S-3.5**, **Supporting Information**). Identified compounds are represented in terms of number of hexoses (H), *N*-acetylglucosamines (N), fucoses (F) and sialic acids (S). The three main glycopeptides from IgG are defined as G0F (H3N4F1), G1F (H4N4F1) and G2F (H5N4F1).Results of direct infusion experiments were processed using LaCyTools, a LC(/CE)-ESI-MS data processing tool based on MassyTools.[202] A sum spectrum was created for each flow rate, using a total time window of 1 min. Sum spectra were calibrated using at least five calibrants with signal-to-noise (S/N) ratio ≥ 9, including the three main glycopeptides G0F, G1F and G2F measured both under doubly- and triply-charged form. Subsequently, the peak area was integrated using 75% of the theoretical isotopic pattern of each analyte, corresponding to the first three isotopes of all glycopeptides with a window of ± 0.1 *m/z* units. The background was measured in a ± 10 Da mass window around each glycopeptide and subtracted from the compound peak area.

Operating conditions for nano-LC-ESI-MS experiments are described in **S-3-1-3**, **Supporting Information**. Experimental procedures for the determination of LODs, repeatability and intermediate precision, as well as evaluation of the influence of the flow rate on MS signal intensity are described in **S-3-1-4**, **S-3-1-5**, and **S-3-1-6**, **Supporting Information**.

RESULTS AND DISCUSSION

This study presents the integration of a DEN-gas setup with the commercially available sheathless CE-ESI hyphenated to a MS system to improve the overall sensitivity and repeatability in glycopeptide analysis. After optimization of ESI source and MS parameters, the performance (*i.e.,* sensitivity, repeatability, and detector response) of the CE-ESI-MS setup equipped with DEN-gas was investigated and compared to the conventional CE-ESI-MS system without DEN-gas. Where possible, figures of merit from a state-of-the-art nano-LC-ESI-MS setup were also included in the method comparison.

OPTIMAL CE-ESI-MS OPERATING CONDITIONS

The technical modifications of the commercial sheathless CE-ESI-MS system, described and illustrated in **Figure S-3.1**, **Supporting Information**, allowed for a straightforward addition of the DEN-gas into the ion source while providing a simple way to change between both setups (*i.e.,* CE-ESI-MS with and without addition of DEN-gas). The best source conditions for the CE-ESI-MS setup were determined using MeCN as DEN-gas, similar to nano-LC-ESI-MS with DEN-gas where MeCN showed optimal performance as a dopant.[195] Optimal parameters were determined based on signal intensities, background noise, in-source fragmentation and repeatability of the relative abundances of glycopeptides during analysis of tryptic fucosylated Fc (fragment crystallizable) glycopeptides from polyclonal IgG1 (**Table S-3.1**, **Supporting Information**). As shown in **Figure 3.1**, the relative peak areas observed for the studied glycopeptides were consistent with or without DEN-gas.[177] Compared to CE-ESI-MS without DEN-gas, an overall higher intensity (*ca.* 2-fold enhancement for all glycopeptides) was observed with the DEN-gas supply (**Figure S-3.8.A**, **Supporting Information**). Moreover, the addition of DEN-gas led to a lower abundance of noise and interferences over the whole MS detection range (*i.e., m/z* 200-2000), especially in the region between *m/z* 500-800 which showed the highest level of interferences in the spectra without DEN-gas (**Figure S-3.9**, **Supporting Information**). Thus, by adding MeCN as solvent vapor during ionization, an overall 2-fold increase in sensitivity (expressed as S/N ratios) was observed (**Figure S-3.8.B**, **Supporting Information**).

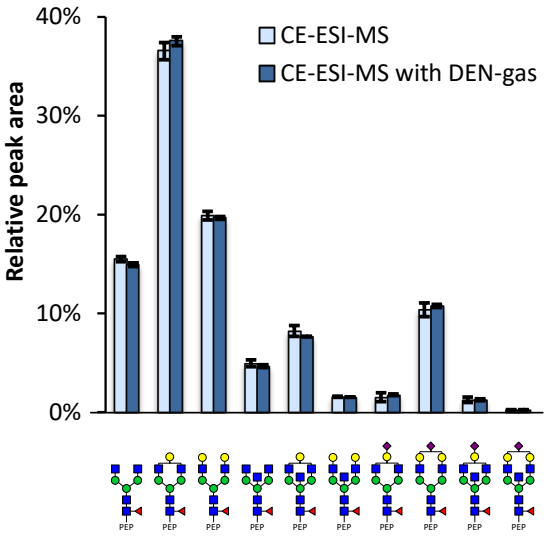

Figure 3.1: Relative peak areas observed for IgG1 glycopeptides with CE-ESI-MS and CE-ESI-MS with DEN-gas setup. Tryptic fucosylated glycopeptides were studied from IgG1. Error bars represent the standard deviation ($N = 3$). PEP illustrates the tryptic peptide sequence EEQY**N**STYR.

The presence of the DEN-gas in the ESI source was found to have a slight effect on the electrophoretic separation, as illustrated in **Figure 3.2**. With the DEN-gas, the observed mobilities were slightly higher, which might be explained by a suction effect caused by the gas flow rate. However, the impact on both separation efficiencies and peak resolution was negligible and did not hamper glycopeptide ionization and quantification.

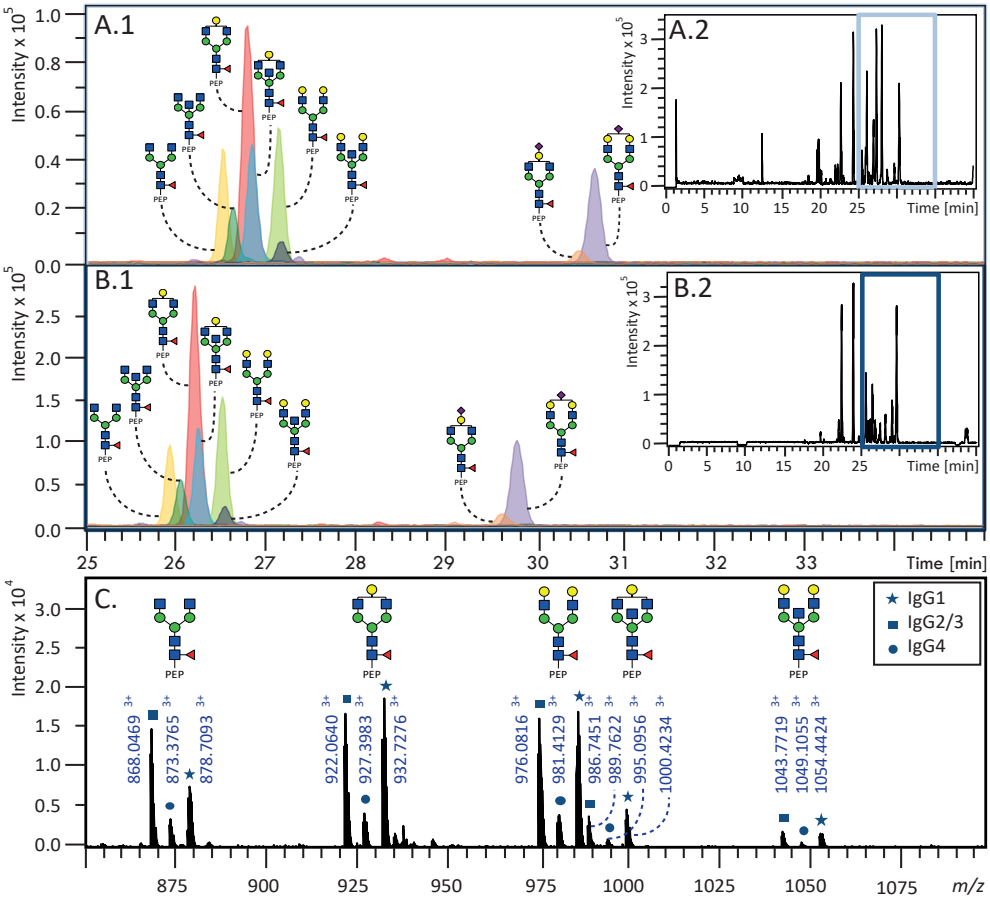

Figure 3.2: Influence of DEN-gas on the EIE of the tryptic Fc *N*-glycopeptides from IgG1. (A) Without DEN-gas and **(B)** with DEN-gas, a sum MS spectrum recorded for the different IgG subclasses between 25.8 and 26.8 min of **Figure 3-2.B** is shown in panel **C**. **A.2** and **B.2** show the base peak electropherograms and highlight the time window extracted for **A.1** and **B.1**.

EFFECT OF DIFFERENT DOPANTS

The effect of the solvent composition (*i.e.,* MeCN, IPA and EtOH) was investigated by comparing the peak areas obtained for fucosylated glycopeptides from IgG1. As shown in **Figure S-3.10**, **Supporting Information**, S/N ratios were significantly higher for all fucosylated glycopeptides with MeCN compared to the other two studied dopants. EtOH and IPA lead to relatively lower intensities than MeCN for most of the glycopeptides. Moreover, a different ionization behavior was observed with EtOH and IPA compared to MeCN with lower relative abundances of sialylated glycopeptides (*i.e.,* G1FS, G2FS, G1FNS, and G2FNS). MeCN was selected for further DEN-gas experiments due to higher intensities, better coverage of sialylated glycopeptides and similarity of the glycosylation profiles with the conventional setup (**Figure 3.1**).

The sensitivity enhancement observed for fucosylated glycopeptides obtained from polyclonal IgG1 was also observed with the analysis of tryptic Fc *N*-glycopeptides from IgGmAb1 where an average 2-fold increase in S/N ratios was measured (**Figure S-3.11**, **Supporting Information**) while the relative abundances of glycopeptides remained constant. Moreover, similar relative abundances were observed with CE-ESI-MS configuration equipped with DEN-gas compared to two state-of-the-art methods, *i.e.,* nano-LC-ESI-MS (tryptic glycopeptides) and HILIC-FLD (PNGase F released *N*-glycans labelled with 2-aminobenzamide), as illustrated in **Figure 3.3**.[203]

Besides sensitivity improvement, a higher charge state was observed for all glycopeptides when using the DEN-gas. This is illustrated in **Figures S-3.12 - S-3.15**, **Supporting Information**, which show the mass spectra recorded for glycopeptides G0F, G1F, G2F and sialylated glycopeptide G2FS. For instance, for glycopeptide G0F (**Figure S-3.12**), more than half of the ions (56%) were present as $[M+2H]^{2+}$ ions with the conventional setup, while a higher

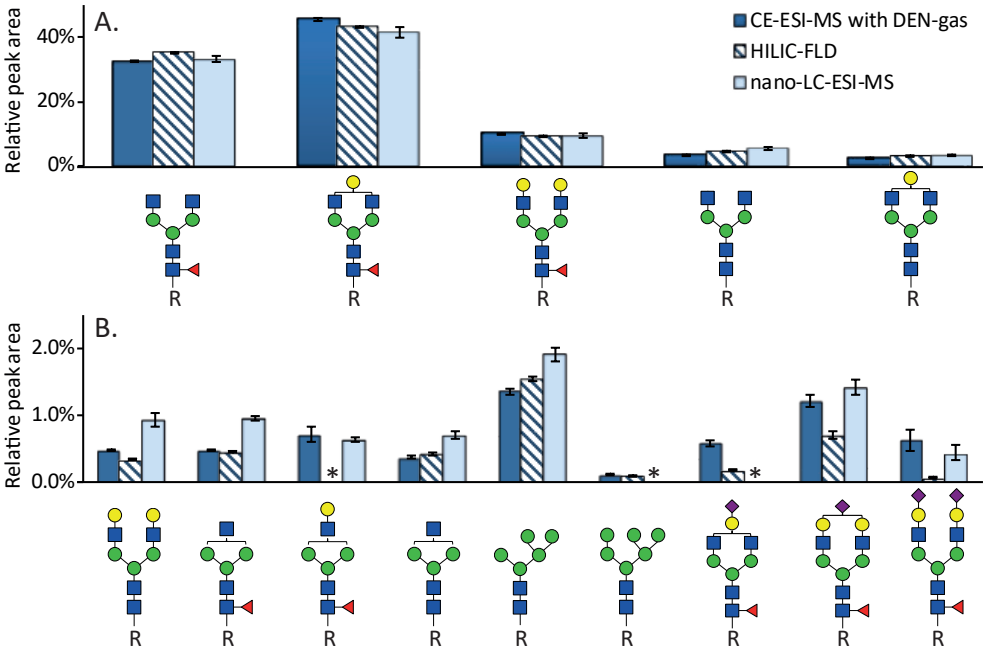

Figure 3.3: Relative abundance observed for IgGmAb1 with CE-ESI-MS with DEN-gas, HILIC-FLD and nano-LC-ESI-MS. Data from HILIC-FLD and nano-LC-ESI-MS was retrieved from Reusch et al.203 Relative abundance was measured for the most abundant glycopeptides **(A)** and low abundant glycopeptides **(B)** on two consecutive days with multiple injections ($N = 6$). Error bars represent the standard deviation. The R attached to the glycan illustrates either the 2-aminobenzamide label (2-AB) on PNGase F released glycans used for HILIC-FLD analysis or the tryptic peptide sequence EEQY**N**STYR for nano-LC-ESI-MS and CE-ESI-MS with DEN-gas. *: glycan form was not detected.

charge state was observed with the DEN-gas supply where the most abundant ions detected were $[M+3H]^{3+}$ (89%). Additionally, the $[M+4H]^{4+}$ ions were observed (0.4%), which were not detected in the conventional setup. Likewise, the glycopeptide G2FS showed a relatively high abundance of ions $[M+4H]^{4+}$ with DEN-gas (14%) than without (0%) (**Figure S-3.15**).

EFFECT OF FLOW RATE ON MS SIGNAL INTENSITY

The influence of the flow rate on MS signal intensity was evaluated for IgGmAb1 glycopeptides via direct infusion over a large range of flow rates (*i.e.,* 1 - 88 nL/min) with and without the presence of DEN-gas. An overall increase in MS signal was observed with the DEN-gas setup regardless to which flow rate was employed, as shown in **Figure 3.4**. A similar increase in signal has been observed for different glycoforms. Notably, a 5-fold increase in signal intensities was found at flow rates hgher than *ca.* 50 nL/min. At these flow rates, both setups showed a concentration-sensitive response (*i.e.,* detector signal is proportional to the analyte concentration, but is independent of the mass flow).[112] The benefits of using the DEN-gas setup in glycopeptide analysis are therefore predominant in this regimen which is known to be more prone to signal suppression effects compared to a mass-sensitive regime (*i.e.,* detector signal is proportional to the mass flow).[189,190] At lower flow rates, the mass flow of the analyte seemed to be predominant when using the DEN-gas resulting in a *ca.* 2- to 4-fold sensitivity enhancement depending on the flow rate. Thus, a mixed regime area was observed between *ca.* 4 and 50 nL/min, which was mainly concentration-sensitive with a slight mass-sensitive contribution.

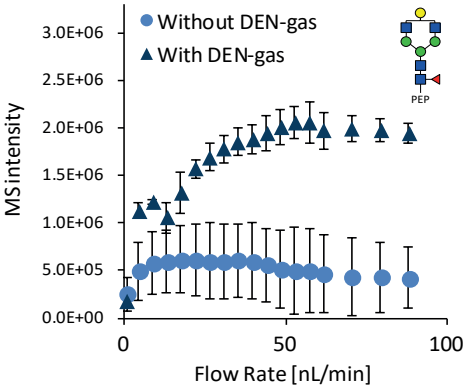

Figure 3.4: MS intensity as a function of the flow rate with and without DEN-gas for glycopeptide G1F of IgGmAb1. Purified IgGmAb1 (0.17 ng/nL) was infused in 10% HAc (*v/v*). The error bars represent the deviation ($N = 3$). Peptide backbone EEQY**N**STYR.

The presence of the DEN-gas led to improved ionization efficiencies over the large range of flow rates studied. The increased gain at higher flow rates suggests that further sensitivity enhancement might be expected for more concentrated samples. Additionally, an enhanced sensitivity can be expected when higher sample volumes would be injected with an on-line stacking procedure, such as t-ITP, increasing the amount of analytes reaching the detector.

EFFECT OF INJECTION VOLUME

CE features the possibility to perform on-line sample pre-concentration within the separation capillary. This approach allows for the injection of more than 50% of the capillary volume (*versus* conventional injection of 1 - 2%) without peak broadening by focusing the analytes into a minimal volume, an approach referred to as sample stacking. The t-ITP approach used in this study relies on the injection of the sample dissolved in AAC, acting as the leading electrolyte, followed by a small post-plug of BGE acting as the terminating electrolyte.[198] When applying the separation voltage, an electric field gradient is established due to the different zone conductivities leading to the stacking of the analytes of interest behind the leading electrolyte, as a function of their respective mobility and the Kohlrausch regulating function.[204,205] The combination of t-ITP with DEN-gas supply was evaluated with different injection volumes (9 - 352 nL, according to Hagen-Poiseuille equation and assuming a BGE viscosity of 1.04 mPa·s, corresponding to 1.4 - 55% of the capillary volume)[112] and compared to the conventional CE-ESI-MS setup.

Figure 3.5 illustrates the increase in peak areas with and without DEN-gas as a function of the injected volume for the IgG1 glycopeptides G1F (**Figure 3.5.A**) and G2FS (**Figure 3.5.B**). For the tryptic Fc *N*-glycopeptide G1F, the ionization behavior showed distinct trends dependent on the volume injected, *i.e.,* a linear relationship at low injected volumes, while a plateau was observed at higher injected volumes followed by a decline in MS intensity. Indeed, when injecting high volumes, *i.e., ca.* 178 nL (corresponding to 28% of the capillary

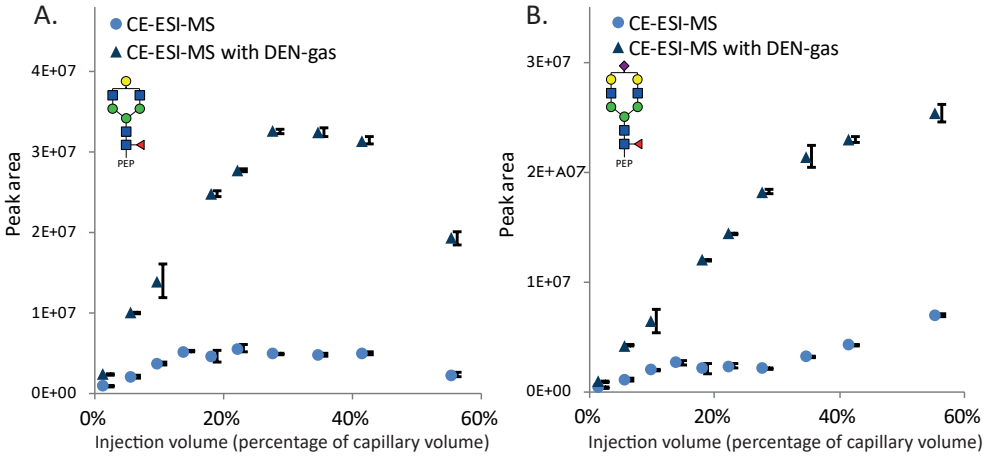

Figure 3.5: Peak areas of IgG glycopeptides G1F (A) and G2FS (B) obtained with conventional CE-ESI-MS and CE-ESI-MS with DEN-gas with different volumes injected. Different volumes injected are expressed as percentage of the total capillary volume. The error bars represent the range ($N = 2$) which are shown with an x-axis offset of 1% from the original data point. PEP illustrates the tryptic peptide sequence EEQY**N**STYR.

volume) with DEN-gas and *ca.* 95 nL (corresponding to 15% of the capillary volume) without DEN-gas, a deleterious effect was not only observed on the MS ionization but also on the CE separation, despite the t-ITP stacking procedure. By injecting higher sample volumes, the separation window became narrower, leading to the co-migration of the non-sialylated glycopeptides with an autolysis product from bovine trypsin, as illustrated in **Figure S-3.16**, **Supporting information**. One may speculate that the presence of this co-migrating autolysis product led to significant ionization suppression of the glycopeptides of interest (**Figure S-3.17**, **Supporting information**), explaining the plateau and subsequent decrease in MS intensity observed in **Figure 3.5.A**. Glycopeptide G2FS showed a slightly different ionization behavior (**Figure 3.5.B**) when increasing the injected volume. The sialylated species were less impacted by co-migration of other analytes (as illustrated in **Figure S-3.18**, **Supporting information**), but injection of larger volumes of samples led to unacceptable peak shapes for sialylated species, as clearly emphasized in **Figure S-3.16**, **Supporting information**, hampering a proper integration of the MS signal.

Overall, these results show that combining the DEN-gas with a relatively large injected volume allowed for a significant improvement in sensitivity compared to the conventional CE-ESI-MS setup. Moreover, adding MeCN vapor during the ionization process allowed for a higher dynamic acquisition range, rendering this approach very promising for quantitative purposes. The combination of the t-ITP pre-concentration technique and DEN-gas represents an attractive approach to reach the highest sensitivities in CE-ESI-MS, provided that a compromise is found between maximum volume injected and optimal separation efficiency.

LIMITS OF DETECTION

LODs were determined for the G2F glycopeptide of IgGmAb1 with both CE-ESI-MS setups and compared to LODs determined for nano-LC-ESI-MS. **Table 3.1** shows the LODs of the lowest concentration that could still be detected with a S/N ratio of ≥ 3. The minimum absolute molar amount detected is additionally translated to total IgGmAb1, factoring in the relative abundance of the G2F glycoform and the glycosylation site duplicity in the Fc-dimer. LODs of 0.9 amol were obtained with CE-ESI-MS with DEN-gas compared to 1.7 fmol with nano-LC-ESI-MS. This improvement is illustrated in **Figure 3.6**, which shows the differences observed in the S/N ratios between the three methods at relatively high, medium, and low concentrations. At relatively low concentrations (**Figure 3.6.C**), no signal was detected with the reference method (nano-LC-ESI-MS) while CE-ESI-MS with DEN-gas led to higher S/N ratios (10-fold) compared to the conventional setup. This higher sensitivity was confirmed when reporting the peak areas, as illustrated in **Figure S-3.19**, **Supporting Information**.

Table 3.1: Limits of detection observed for IgG and total glycopeptides in nano-LC-ESI-MS and CE-ESI-MS with and without DEN-gas.

	IgG concentration (pg/µL)	IgG injected amount (pg)	IgG molar amount (amol)	Total glycopeptides molar amount (amol)
Nano-LC-ESI-MS	250.0	250.0	1700.0	320.0
CE-ESI-MS	75.0	3.3	22.0	4.4
CE-ESI-MS with DEN-gas	3.0	0.1	0.9	0.2

CE-ESI-MS is usually considered to have a lower concentration sensitivity than chromatographic approaches due to the significantly lower amount of sample that can be injected (nL *versus* µL range). However, by combining the sheathless approach with DEN-gas supply and on-line pre-concentration techniques, CE-ESI-MS showed better concentration sensitivity than the state-of-the-art nano-LC-ESI-MS approaches, making it a very competitive and attractive technique for glycopeptide analysis.

REPEATABILITY AND INTERMEDIATE PRECISION

Compared to chromatographic techniques, CE-ESI-MS is usually considered less repeatable, especially when using the sheathless approach due to a lower stability of the ESI interface.[206] The effect of the DEN-gas supply on repeatability and intermediate precision was investigated at low, medium, and high concentrations for the three most abundant tryptic glycopeptides from IgGmAb1. **Tables 3.2** and **3.3** report the results on relative peak

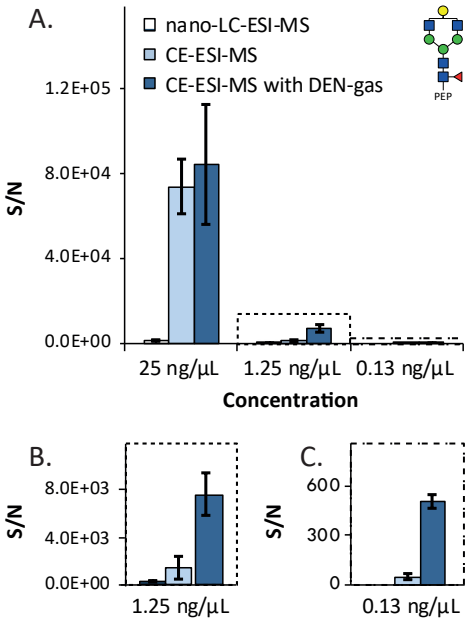

Figure 3.6: Signal-to-noise ratio (S/N) of IgGmAb1 glycopeptide G1F obtained with nano-LC-ESI-MS, conventional CE-ESI-MS, and CE-ESI-MS with DEN-gas at different concentrations. For CE-ESI-MS experiments, samples were further diluted with leading electrolyte (3:2, *v/v*) prior to injection. **(A)** S/N observed at relatively high, medium, and low concentrations. Magnifications of the medium and low concentration are displayed in **(B)** S/N observed at 1.25 ng/µL and **(C)** S/N observed at 0.13 ng/µL. This concentration was below the LOD of nano-LC-ESI-MS and hence G1F was not detected (*). Error bars represent the standard deviation ($N = 3$). The PEP illustrates the tryptic peptide sequence EEQY**N**STYR.

areas observed for repeatability (intraday variability) and intermediate precision (interday variability), respectively. Relative standards deviations (RSDs) were lower than 4% for all studied concentrations, which is similar to results reported for nano-LC-ESI-MS.[207] A high repeatability is especially beneficial in profiling research, where an RSD of maximum 5% is generally accepted in the glycoproteomics field.[203,208] This repeatability is for instance essential in the development of biopharmaceuticals and analysis of clinical biomarkers, since differences in glycosylation are known to have a significant impact on conformation, stability, and function of glycoproteins.[209,210]

EFFECTS OF DEN-GAS SUPPLY ON OTHER GLYCOPEPTIDES

The investigation of CE-ESI-MS with DEN-gas supply was further extended with two additional glycoproteins, namely haptoglobin (Hp) and prostate-specific antigen (PSA). Hp has four *N*-linked glycosylation sites, resulting in three glycopeptides after tryptic cleavage with one glycopeptide containing two *N*-linked glycosylation sites. Using the DEN-gas supply, the most abundant glycopeptides showed relative peak areas that were very similar to those observed without DEN-gas (**Figures S-3.20.A, S-3.21.A**, and **S-3.22.A**, **Supporting Information**). However, different improvement factors of S/N ratios and absolute peak areas were observed depending on the peptide backbone. For instance, glycosylation site $\mathbf{N}_{184}$ has a peptide backbone of 24 amino acids and showed a *ca.* 11-fold increase in peak area and *ca.* 31-fold increase in S/N ratios (**Figure S-3.20**, **Supporting Information**), while the peptide with glycosylation site $\mathbf{N}_{241}$ (16 amino acids) showed a *ca.* 4-fold increase in peak

Table 3.3: Repeatability of tryptic Fc *N*-glycopeptides from IgGmAb1 with CE-ESI-MS using the DEN-gas. Relative abundances (normalized to the sum of these three glycoforms) for low, medium and high concentrations are shown with their relative standard deviation *(N = 3)*.

	Relative abundance		
Concentration (ng/µL)	**G0F**	**G1F**	**G2F**
0.03	37% (3.2%)	51% (2.6%)	12% (1.5 %)
0.30	37% (1.1%)	51% (0.9%)	12% (2.7%)
3.00	37% (0.6%)	52% (0.4%)	11% (1.0%)

Table 3.2: Intermediate precision of tryptic Fc *N*-glycopeptides from IgGmAb1 with CE-ESI-MS using the DEN-gas. Relative abundances (normalized to the sum of these three glycoforms) for low, medium and high concentrations are shown with their relative standard deviation (*N = 3*).

	Relative abundance		
Concentration (ng/µL)	**G0F**	**G1F**	**G2F**
0.03	38% (1.5%)	50% (1.5%)	12% (1.7 %)
0.30	38% (1.2%)	51% (0.7%)	11% (5.5%)
3.00	37% (0.1%)	52% (0.3%)	11% (1.1%)

area and *ca.* 6-fold increase for S/N ratios (**Figure S-3.21**, **Supporting Information**). For the glycopeptide with 13 amino acids and two potential glycosylation sites, namely $\mathbf{N}_{207}$ and $\mathbf{N}_{211}$, the H5N4S1 glycoform showed a 2-fold increase in peak area and S/N ratio, while H10N8S2 gave a 3-fold increase in peak area and a 2-fold increase in S/N ratio (**Figure S-3.22**, **Supporting Information**). PSA, which contains a single *N*-linked glycosylation site, resulting in a peptide backbone of two amino acids ($\mathbf{N}_{69}$K) after tryptic cleavage, showed a 2-fold and 3-fold enhancement in absolute peak area and S/N ratio, respectively (**Figure S-3.23**, **Supporting Information**).

All studied glycopeptides displayed similar relative ratios between the CE-ESI-MS-platform with and without DEN-gas. When using the DEN-gas supply, an overall gain in sensitivity (*i.e.,* 2- to 31-fold increase) was observed for diverse glycopeptides with different peptide backbones, glycan structures and glycosylation sites, where singly-glycosylated peptides showed higher sensitivity improvement than non-glycosylated species.

MECHANISTIC CONSIDERATIONS

The combination of the DEN-gas setup with the commercial sheathless CE-ESI-MS system showed significantly higher sensitivities and improved repeatability for glycopeptides analysis. The positive impact of adding a dopant to the gas-phase may be explained by the combination of complex and multimodal mechanisms acting on both evaporation and ionization. For instance, Nguyen and Fenn already showed that the addition of polar solvent vapors to a nitrogen sheath gas led to a substantially higher abundance of desolvated ions and, thus, a higher intensity.[191] The enthalpy of condensation, released by adsorption of the solvent vapor molecule to the droplet surface, might lead to the formation of “hot spots” which help nearby solvent molecules to evaporate from the surface. Another mechanism may be linked to the proton transfer occurring in the gas-phase in presence of a solvent. The difference in both ionization efficiencies and charge state distributions may be explained by the different gas-phase basicity and proton affinity of MeCN present in the DEN-gas compared to water.[211] Other relevant mechanisms may contribute to these observations and further research is necessary to enable the prediction of glycopeptides ionization behavior in the presence of a DEN-gas.

CONCLUSION

This study evaluated the integration of a DEN-gas supply with sheathless CE-ESI-MS for glycopeptides analysis. An overall improved ionization efficiency was found using the DEN-gas which is in line with previous findings of nano-LC-ESI-MS measurements. The optimized sheathless CE-ESI-MS setup equipped with DEN-gas and using MeCN as a dopant showed improved sensitivities for all the glycopeptides studied compared to the conventional CE-ESI-MS setup. Notably, by combining an on-line pre-concentration technique and an optimal flow rate with the DEN-gas, a considerable factor of improvement was reached. LODs were found to be significantly lower than observed with state-of-the-art nano-LC-ESI-MS. Moreover, the usage of DEN-gas showed an excellent repeatability with RSDs lower than 4%, showing its benefits in protein glycosylation analysis.

Overall, this study presents a powerful analytical tool for glycopeptide analysis with an enhanced sensitivity and repeatability. Further investigations are needed to better understand the complex and multimodal mechanisms underlying the observed sensitivity improvement (*e.g.,* using a larger set of compounds). Additional perspectives of this work include the investigation of ionization tuning via addition of modifiers to the dopant, for instance a base or acid, and variation of the dopant concentration. Moreover, the effect of DEN-gas in metabolomic or proteomic applications remains open and needs to be evaluated. Notably, the addition of DEN-gas to a CE-ESI-MS setup may be beneficial in proteomics, allowing for the addition of supercharging agents (*e.g.,* m-nitrobenzyl alcohol, sulfolane, etc.) to MeCN, leading to detection of higher charge states of peptides and resulting in a higher number of identified peptides.

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SUPPORTING INFORMATION

The full supplementary information of this thesis can be found via *https://doi.org/10.17026/dans-zg4-nxca*. In addition, the supporting information as refered to in G.S.M. Kammeijer *et al.*, "*Dopant Enriched Nitrogen Gas Combined with Sheathless Capillary Electrophoresis–Electrospray Ionization-Mass Spectrometry for Improved Sensitivity and Repeatability in Glycopeptide Analysis*", Analytical Chemistry 88 (11), pp 5849-5856, 2016, is available via *https://pubs.acs.org/doi/suppl/10.1021/acs.analchem.6b00479.*