EXPLORING THE PROTEOME BY CE-ESI-MS

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Introduction

The success of the genome sequencing projects in the late 90's and early 2000's boosted enthusiasm and hope for easy implementation of the genomic approach to other levels of biological regulation[1]. The totality of an organisms proteins, known as the proteome, appeared as a logical next target. However, the human genome showed the task of identifying over 100,000 proteins controlled by 22,000 protein coding genes to be an enormous logistical and technological challenge. Developments in the fields of mass spectrometry and to a great extent data processing tools have helped many field of biological analysis but none-more so than the field of proteomics. The mass spectrometric ability to select a specific mass to charge ratio and obtain fragments from this precursor in tandem mass spectrometry experiments allows for identification of the molecular structure and in the case of a peptide the amino acid sequence. A human sample can potentially containing up to 100,000 proteins which makes identification of the individual proteins very complex. The problem is further exacerbated by the fact that proteins can be present in concentrations scaling many orders of magnitude and, opposed to genomic analysis, no amplification tools to identify low concentration proteins are available. For this reason mass spectrometry needs to be coupled to a separation technique which allows for concentration of samples before detection of very low concentration proteins but also for de-complexing the sample before it reaches the mass spectrometer to obtain more reliable spectra. Developments in separation techniques are providing more and more separation power but the ionization techniques for the coupling of these separation techniques to MS are also still a major field of development.

Ionization in mass spectrometry

The first reports of what is now called mass spectrometry were made as early as 1886 by E. Goldstein and consisted of what he called rays of positive electricity[2]. This was the first documented observation of positive gas-phase ions that were created using a high voltage anode, which formed the basis of the rapid development and application of mass spectrometry in the following century.

The three most commonly used ionization techniques for coupling a separation system to mass spectrometry are Electron (impact) Ionization (EI)[3], Chemical Ionization (CI)[4] and Electrospray Ionization (ESI)[5]. Of these three EI is by far the oldest technique, discovered in 1918 by A.J. Dempster and used then in his research to create gas phase ions from solids. A short time later Tate and Smith applied the technique in the ionization of gasses and vapors[6]. The principle of EI works through bombarding the gas phase analytes that are coming off the separation column. Ions are then assumed to be formed through the $M + e^- = M^{\cdot +} + 2e^-$ principle resulting in molecular ions that have the mass of the uncharged

analyte minus the mass of an electron, which compared to the mass of a proton and therefore molecules is negligible. Electron ionization is a relatively harsh ionization technique which produces a large amount of in source fragmentation. This fragmentation can be useful for identification purposes but when analyzing a complex or very depleted sample it does reduce the sensitivity and specificity through a significant loss in parent ion intensity. Currently, it is still the most commonly used ionization technique in gas chromatography (GC) coupled to MS. Applications of EI in liquid chromatography to MS coupling are limited as there are very powerful alternatives[7]. The source conditions in GC-MS are more suitable for EI because of the vacuum conditions already existing inside the source. To obtain very low pressure/vacuum conditions in LC-MS analyses requires a great deal more effort as the LC will push large amounts of fluid turning into gas into the source and thereby continually counterbalancing the efforts of the vacuum pumps to create a low pressure system.

Chemical ionization as discovered by Munson and Field in 1966 employs a reagent gas that is continually pumped in the MS source. The reagent gas is ionized through bombardment with electrons from a high voltage electrode most commonly in the form of a filament present in the source. An ion is created after the collision of an analyte molecule with ionized reagent gas. Methane, ammonia, and isobutene are some of the reagents that are commonly used in CI. As the reagent gas is present in large excess compared to the analytes, the electrode will mostly be ionizing the reagent gas instead of directly ionizing the analytes by EI. Chemical ionization does not occur under vacuum conditions as it requires the presence of significant concentrations of reagent gas to function properly. Chemical ionization as described above is mostly used in GC-MS analysis. Compared to EI, CI is a much softer ionization technique as it imparts much less residual energy onto the target analyte thereby strongly reducing the amount of fragmentation that occurs during the ionization process.

Shortly after the development of CI an adjustment to the setup resulted in what is now called atmospheric pressure chemical ionization (APCI). Where CI is only suited for GC-MS coupling as it requires low source pressure, APCI is more suitable for LC-MS coupling. In APCI-MS of liquid samples, the eluent is introduced into a pneumatic nebulizer where the solvents carrying the analytes are dispersed into a thin fog through a high temperature nitrogen flow. The analytes in the resulting small droplets are carried through a desolvation chamber to obtain gas phase analytes before entering the MS source. The stream of gas passes by a corona discharge needle that replaces the CI filament to bombard the reagent gas with electrons to create the primary ions. The

adjustments to the original CI approach, including the change in pressure at which the source operated, resulted in the new name atmospheric pressure chemical ionization (APCI).

The development of APCI allowed for easier coupling of liquid phase (mostly LC) separations to mass spectrometry as the desolvation stage of the APCI source was capable of coping with the high flow rates (mls/min) that were typically used for LC in the 70's and 80's. Currently, CI and APCI are still regularly used in both GC- and LC-MS analysis. However, with the discovery of electrospray ionization (ESI) and the developments in ESI source design for LC-MS at higher flow rates APCI is not the most predominant ionization strategy. The selection of varying reagent gases can give (AP)CI more specificity compared to EI and electrospray ionization making it the go to method for certain applications[8].

The final and most commonly used ionization method is ESI which was first reported by Dole et al.[9] and later by the group of John B. Fenn[5, 10, 11] and in parallel by Alexandrov et al. in 1984 [12]. ESI is performed at or just below ambient pressure by flowing solvent (or eluent) containing analytes through a needle. A potential difference is applied between the ESI needle and the source inlet resulting in a charge driven spray. Two separate approaches can still be found in source designs of the varying mass spectrometry manufacturers where one approach is the grounding of the needle and applying a voltage on the source inlet to obtain the required potential difference. The second and more prevalent approach is the application of an electrospray voltage to the spray needle (1 kV to ± 6 kV range) and a grounded source inlet which also provided the required potential difference. The early ESI sources were operated in a fashion very similar to what today is called nano-electrospray. The application of a counter flowing dry gas allowed for the analysis of increasingly bigger molecules[10]. The flow rate of the sprayed solution was in the low microliter per minute range which was problematic as liquid chromatography flow rates were still in the milliliter per minute range.

Ultra-low flow Electrospray

At the time of the discovery of ESI capillary electrophoresis was already employing flow rates in the nano liter per minute range making it a suitable candidate for coupling to mass spectrometry. Smith and Udseth developed the coaxial sheathliquid interface to operate in this low microliter flow rate range [13, 14] and to date this sprayer is still the most commonly used approach for coupling CE to MS detection. In the 90's great strides

were made in the development of LC and this dramatically decreased the operating flow rates from milliliters to microliters and then even down to the high nanoliter per minute range in the case of nano-LC. This allowed for easier coupling to mass spectrometry especially in sources that were using nebulizer assisted and heated sprayers to obtain better evaporation of higher flow rates. In 1994 Wilm & Mann described the application of nano-electrospray which was able to produce a stable electrospray at flow rates of 25 nl/min. The coupling of CE to mass spectrometry at these lower flow rates was not as successful and despite a large amount of research being spent on developing the proper interface design for sheathless CE-ESI-MS interfacing no commercial set up was ever made available and thus this approach never found mainstream use[15].

Nano-electrospray quickly found interest for the coupling of nano-scale separations like nano-LC to mass spectrometry for the analysis of limited amounts of sample and thereby providing optimal sensitivity from low required amounts of starting sample. The effects of decreasing flow rates on electrospray ionization have been investigated over the years. It was shown that at lower flow rates (<100) the signal intensity is not completely concentration dependent but actually decreases with decreasing flow rate[16]. This was named the mass sensitive regime indicating that the signal intensity is related to the absolute amount of material that is put into the system and no longer to the concentration of analyte in the sample[17].

Schmidt et al. found that at decreasing flow rates signal intensity ratios could change as a result of improved ionization efficiency[18]. They postulated that ion suppression effects could be overcome at very low flow rates as almost complete evaporation and ionization could be accomplished. Flow rates that are categorized as ultra-low flow are those that fall within the range required to reach the mass sensitive electrospray regime. The field of CE was introduced to the first robust manner to couple CE separations to mass spectrometry at very low separation flow rates with the development of the porous sheathless interface by Moini[19]. This interface has shown to provide stable electrospray at flow rates down to 4.5 nl/min. These flow rates are well within the mass sensitive regime and in the flow rate range that Schmidt et al. showed to present altered ionization efficiency for certain compounds.

Low flow CE-MS

The use of ultra-low flow rates or even stagnant separation medium conditions in CE separations is not uncommon and when applied in the right way can result in significant

increases in electrophoretic separation power. There are even instances of using reversed flow, either by specific coatings causing electro-osmotic flow (EOF) or by pressure, to obtain optimal separation for targeted analysis.

A drawback of coupling CE to mass spectrometry through previous sheathless approaches was the requirement of a significant linear flow through the separations system. This was due to bubble formation at the outlet electrode resulting in intermittent spray or even separation current breakdown[15]. The linear flows that are required for these separations to take place significantly reduce the separation time but thereby severely limit the overall separation power of the system.

Using the co-axial sheathliquid interface and specifically tailored separation conditions it is possible to perform separations at very low or even non-flow conditions resulting if very high potential separation power. However, the well-known drawback to the co-axial interface is the dilution effect that is experienced in the ionization process. Especially when employing separation flow rates in the low nano-liter range the dilution factor can run into the thousands[15].

The porous sheathless interface has the ability to produce stable electrospray at ultra-low separation flow rates[17]. Separations in these capillaries result in very high resolving power combined with very high sensitivity in the electrospray process. A further advantage of ultra-low flow rates in the separation system is the potential for loading significant sample volumes in combination with stacking techniques. As the ultra-low flow rates result in long analysis times there is sufficient time for the stacking processes to take place before separation occurs and before the analytes reach the MS.

CE-MS in bottom-up proteomics

Currently, bottom-up proteomics is the most developed strategy in the field of protein analysis, which requires the proteolytic digestion of proteins before analysis and identification of measured peptides on basis of already developed protein amino acid sequence databases. Although proteolytic digestion is not the ideal approach as it ads extra complexity to samples that could potentially contain tens of thousands of proteins to start off with, technology allowing for easy analysis of intact proteins is limited and not generally available. Bottom-up proteomics samples are of such complexity that simple infusion into a mass spectrometer would not provide enough identification power to cover more than the most abundant part of the proteome. At present, the combination

of nano-reversed phase liquid chromatography (RPLC) and mass spectrometry is the most commonly applied strategy in bottom-up proteomics analysis. As nano-RPLC was the first separation technique that could provide high peak capacity separations at low flow rates and could be coupled to mass spectrometry through nano-electrospray it was the obvious choice in many labs[20]. Due to the issues described in the previous section the sheathless coupling of CE to mass spectrometry was not possible and combined with the limited loadability of CE in general it could not compete with the performance of nano-RPLC-MS with regard to absolute sensitivity. The recent development of the porous sheathless interface and the electro-kinetic junction interface have re-sparked the interest of the bottom-up proteomics field for the potential of CE separations. Well over 30 papers have been published in which either of these interfaces have been used in bottom-up proteomics approaches. Recent papers have shown the competitiveness of CE-MS in the bottom-up proteomics field with regards to separation power, sensitivity and resulting peptide and protein identifications[21-23]. Furthermore, the identification of shorter and more hydrophilic peptides by CE-MS compared to RPLC-MS has shown the strong complementarity of the two techniques in bottom-up proteomics. CE-MS has also shown capabilities that cannot be matched by liquid chromatography in any form by sequencing the full amino acid content of a monoclonal antibody[24].

CE-MS of restricted sample amounts

Capillary electrophoresis is a technique best known for its very low sample requirement. The low sample requirement comes hand in hand with the very limited loadability. Although a number of sample loading techniques have been developed to significantly improve the loadability of a CE separation system the general potential of sample loading is still only up to a few % of the total sample amount. Improving the percentage of loaded sample is predominantly a technical issue as the commercially available systems from the three biggest CE manufacturers still require a few microliters and even up to tens of microliters for successful injection. This, while at the same time the maximum injection volume in many separation systems is in the tens of nanoliters up to a few hundred nanoliters.

The group of Sweedler has however developed a stainless steel microvial injection system that can effectively inject from sample volumes down to hundreds of nanoliters[25]. Although the technique has not been incorporated in any commercial setup it has the potential to significantly improve CE-MS overall sensitivity as samples could be concentrated down to smaller volumes, thereby overcoming the limited volume

loadability.

The combination of developments in CE-MS interfacing and CE injection technology has resulted in a number of performed proof-of-principle studies on the analysis of very low sample amounts in CE-MS. Faserl et al. have shown that with an order of magnitude less material than used in nano-RPLC-MS similar numbers peptides could be identified in bottom-up proteomics[21]. The Dovichi group have shown that with amounts of material down to 1 nanogram still hundreds of peptides and proteins could be identified from a diluted E. coli digest[26].

These examplse prompt the question whether it would be possible to use CE-MS on a truly material limited sample like laser microdissected (LMD) organ substructures (glomeruli, islets of Langerhans etc.) or even free flowing tumor cells in blood or other bodily fluids. In practice only a few applications of analysis of LMD material have been described and were predominantly performed using nano-liquid chromatography. Waanders et al. showed successful proteomics analysis of LMD pancreas islets of Langerhans by nano-RPLC-MS and could identify thousands of proteins[27]. The transfer of the methodologies that are employed in sample preparation for nano-RPLC-MS analysis to methods suitable for CE-MS analysis is not straightforward. The solid phase extractions (SPE) that are regularly employed for removing reagents and additives (surfactants or chaotropes) that are needed for protein denaturation, reduction and alkylation would result in the loss of many of the strongly hydrophilic and smaller peptides for which capillary electrophoresis is typically suited. For this reason an alternative approach would need to be taken with regard to sample preparation requiring the use of low or no salt additives, potentially volatile or neutral reagents which will not interfere in separation, and omitting a desalting step.

Scope of this thesis

The development of the porous sheathless interface by Moini[19] and the demonstration of its potential to create stable separations and electrospray conditions at ultra-low flow rates [17] warranted significant additional investigation. This thesis can be subdivided in two sections: (1) The investigation of the porous sheathless interface and its potential for improving both ionization and separation **(Chapter 1 - 3)**, and (2) the investigation of the potential of CE-MS and specifically porous sheathless interfacing in bottom-up proteomics. **(Chapter 4 -7)**

To build upon the work of Schmidt et al.[18] the porous sheathless interface was used to produce electrospray at flow rates down to 4.5 nl/min and the effect of such low flow rates on the ionization of phosphorylated peptides was investigated. This showed that significant changes in ionization occur at ultra-low flow rates in the phosphopeptide ionization and that these effects can be utilized in phophoproteomics approaches. **(Chapter 1)** As the porous sheathless interface showed huge potential for highly sensitive detection of peptides the approach was applied in the analysis of IgG1 N-glycosylated peptides. It was found that though the CE-ESI-MS method was a factor 5 slower a 40 fold improvement in sensitivity was achieved when compared to the standard LC-MS platform. The samples obtained in many studies are precious and limited and with an easy buffer transfer the CE-ESI-MS system was able to obtain profiles from sample deemed too dilute for LC-MS analysis. **(Chapter 2)**. Despite all the developments in CE separations since its invention, loadability and separations power have always been at odds with each other. Although iso-electric focusing allows for near complete filling of the separation capillary it has not yet found its way to general applications in CE-MS. With the potential of the porous sheathless interface to perform separation at ultra-low flow rates the question could be raised whether it would be possible to perform separations under zero-flow conditions, thereby optimizing the use of the separation capillary. **Chapter 3** describes the investigation of the use of zero-flow separation combined with high volume sample loading using transient isotachophoresis.

Before any bottom-up proteomics investigation can be developed on basis of CE-MS a thorough understanding of the current technology, required technical aspects and current trends in applications must be acquired. **Chapter 4** describes the technology used in CE-MS bottom-up proteomics in the applications that were published from 2007 to 2012. Both CE-ESI-MS and CE-MALDI are covered, as well as CE sample fractionation techniques and online couplings of CE and LC separations. **Chapter 5** covers CE-ESI-MS bottom-up proteomics applications which utilized the three leading CE-ESI-MS interfaces. This roughly covers the time period 2009 to 2014 although some earlier publications are referenced if so required. **Chapter 5** also covers the technical aspects that are required in the development of a CE-ESI-MS bottom-up proteomics method. It is meant as a starting point for researchers venturing into the field of CE-ESI-MS bottom-up proteomics. **Chapter 6 and 7** describe the application of sheathless CE-ESI-MS in in-depth bottom-up proteomics of fractionated samples (E. coli and human glomeruli respectively). Finally, **Chapter 7** describes the application of a sample preparation method that was specifically tailored to CE-ESI-MS in the analysis of laser micro dissected human glomeruli.

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Chapter 1

Ultra-Low flow ESI-MS for improved ionization efficiency in phosphoproteomics

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Abstract

The potential benefits of ultra-low flow ESI ionization for the analysis of phosphopeptides in proteomics was investigated. Firstly, the relative flow dependent ionization efficiency of non-phosphorylated vs. multiplyphosphorylated peptides was characterized by infusion of a 5 synthetic peptide mix with none to four phophorylation sites at flowrates ranging from 4.5 to 500 nL/min. Most importantly, similarly to what was found earlier by Schmidt et al., it has been verified that at flow rates below 20 nL/min the relative peak intensities for the various peptides show a trend toward an equimolar response, which would be highly beneficial in phosphoproteomic analysis. As the technology to achieve liquid chromatography separation at flow rates below 20 nL/min is not readily available a sheathless CE-ESI-MS strategy based on the use of a neutral separation capillary was used to develop an analytical strategy at flowrates as low as 6.6 nL/min. An inline preconcentration technique, namely transient isotachophoresis (t-ITP) to achieve efficient separation while using larger volume injections (37% of capillary thus 250 nL) was incorporated to achieve even greater sample concentration sensitivities. The developed t-ITP-ESI-MS strategy was then used in a direct comparison with nano-LC-MS for the detection of phosphopeptides. The comparison showed significantly improved phosphopeptide sensitivity in equal sample load and equal sample concentration conditions for CE-MS while providing complementary data to LC-MS, demonstrating the potential of ultra-low flow ESI for the analysis of phosphopeptides in liquid based separation techniques.

1 Introduction

The introduction of modern ESI sources[1-4], and subsequent hyphenation of liquid chromatography[5] (LC) and capillary electrophoresis[6-8] (CE) to mass spectrometry (MS) are probably the most important events in the history of analytical/bio-analytical sciences over the last twenty years. Currently ESI-MS is the core of multiple proteomics workflows, which implies the use of a broad range of LC systems, columns and separation modes over a wide range of flow rates. ESI is indeed compatible with different flow rates, but the technique is inherently optimal at low flow. In early days coupling to HPLC (then > mL/min) was realized through splitting[9]. With the development of nano-ESI[10] and nano-LC such advantages of low flow rates as reduced ion-suppression and improved ionization efficiency became commonly accepted[11-15]. Schmidt et al.[13] have systematically studied the effects of flow rates on the ion signal for a model compound mixture and demonstrated that the ionization bias towards neurotensin, which is commonly observed at usual flow rates, was greatly reduced at flow rates below 20 nL/min. While this is a very important bottleneck for various kinds of MS applications, the mentioned ionization bias is especially problematic in the field of phosphoproteomics, where MS analysis suffers from low ionization efficiency of phosphorylated peptides on top of the already low natural abundance of phosphorylations. Despite much discussion and research on the reasons for the low phosphopeptide ionization efficiency[16-19], improved ionization efficiency only shows a concentration sensitive increase of signal, which results in an equal increase of phospho and non-phospho peptide signal and does not improve the detection of phosphopeptides specifically. For this reason, off-line enrichment techniques have been developed to compensate for the generally high limits of detection in phosphopeptide analysis, but such techniques, while they remain very powerful for large-scale proteomic studies, are costly and labor intensive[20]. Additionally, as any step of an analytical workflow, especially when affinity reactions are involved, they may introduce biases and losses depending on the solid phase material chosen. Another way to potentially improve the compatibility of common workflows for phosphorylated peptides is to work directly on the ESI process where a homogenization of the ionization efficiencies could potentially advance the analysis of phosphopeptides and phosphoproteomics in general. As previously demonstrated, working on the magnitude of the flow rates, rather than or in addition to various enrichment procedures, upstream from the ionization process could represent a very valuable path to explore. Considering LC-based technologies first, which are today the preferred separation strategies in proteomics, it is not an easy task to conduct LC separations at flow rates

below 20 nL/min with satisfactory robustness and high peak efficiencies. In this context, capillary electrophoresis (CE) strategies [21-25] in combination with the ESI sources capable of maintaining a stable spray at such low flow rates[10, 14, 26] may constitute a very interesting option.

Therefore, we have investigated the effect of ultra-low flow ESI for the mass spectrometric detection of phosphopeptides. An ESI interface based on the design of Moini et al.[27] was used to achieve stable ESI spray at flow rates below 10 nL/min. The effects of ultra-low flow on the ionization efficiency of a model peptide with up to 4 phosphorylation sites was studied under hydrodynamic infusion conditions. As a proof of principle, a typical model sample for phosphoproteomics of relatively low complexity was studied. The tryptic digest of bovine milk was analyzed by sheathless CE-ESI-MS using a neutrally coated capillary and the integration of transient-isotachophoresis (tITP) as the sole sample concentration step, without any of the commonly used off-line phosphopeptide sample pre-concentration techniques. Subsequently, the developed strategy was used for the identification of phosphopeptides in a skimmed milk digest sample on an ion trap and compared to results obtained with a “more traditional” phosphoproteomic nano-LC-MS method.

2 Materials and Methods

2.1 Chemicals

All chemicals used were of analytical reagent grade and obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) otherwise stated specially. A 13 amino acid peptide was designed (YQTYPIYASYHLR) with four incorporated tyrosine moieties allowing for its synthesis in a non to tetra-phosphorylated form using a previously reported method[28]. Purity after synthesis was determined with HPLC-UV with the consecutive phosphorylation states being 100%, 100%, 64.7%, 49.9% and 60.2% pure respectively. The measured purities were used for compensation when making stock solutions of the individual synthetic peptides. Powdered bovine milk was purchased locally. All buffers and solutions were prepared in nano-pure water from an Alpha-Q Millipore system (Amsterdam, The Netherlands).

2.2 Sample preparation

Dry milk powder (20 mg) was resuspended in 5 mL 50 mM TEAB (triethylammonium bicarbonate buffer, pH 8.0, Fluka) containing 0.1% of RapiGest (Waters, Milford, MA). The cysteines were reduced with 2 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride) for 45 min at 60 °C and subsequently alkylated with 4 mM MMTS (S-methyl methanethiosulfonate, Fluka) for 30 min at room temperature. Proteins were then digested with trypsin overnight at 37 °C (Sequencing grade modified trypsin, Promega, Madison, WI) using a 50:1 (protein:trypsin) ratio. RapiGest was cleaved and removed from the sample according to the manufacturer's protocol. Aliquots containing 1 mg of the digested sample were lyophilized and stored at -20 °C prior to use.

2.3 Capillary Electrophoresis

All CE and infusion experiments were performed using a PA 800 plus capillary electrophoresis (CE) system from Beckman Coulter (Brea, CA, USA), which was equipped with a temperature controlled sample tray, capillary cooling liquid and a power supply able to deliver up to 30 kV. Both neutrally coated and bare fused silica capillaries were used depending on the requirements per experiment. The neutral capillary coating was a bi-layer with the outer surface consisting of polyacrylamide, currently in development by Beckman Coulter (Brea, CA, USA).

The BGE and LE consisted of 10% acetic acid and ammonium acetate (pH = 4 and various ionic strengths) respectively. Injection volumes were calculated using the Poiseuille equation and a fluid viscosity of 1.04 cP.

2.4 CE-ESI-MS sheathless interface

The detection end of the separation capillary was etched using hydrofluoric acid, creating a porous section of approximately 3 cm following a previously described method[26, 27]. The resulting spray tip has an outer diameter around 40 μm without having a tapered internal diameter. The etched portion of the capillary was inserted into a stainless steel housing containing a retractable head to protect the protruding tip (about 5 mm). The stainless steel housing can be automatically filled with a conductive buffer to close the separation circuit and/or apply the ESI voltage or ground when required. A retractable head was placed around the stainless steel housing as protection of the spray tip. The complete housing was fitted in a custom mount (Beckman Coulter, Brea, CA) to fit the mass spectrometer, which included an x-y-z platform to allow for position optimization. Generally the spray tip was placed coaxially to the MS entrance at a distance ranging from 2 to 5 mm. The positioning of the tip with respect to the MS entrance was optimized by hydrodynamically and/or electrophoretically infusing a test mixture and following the response (intensity and ratio's). The produced interface presented an ability to produce stable ESI sprays at ultra-low flow rates. During long runs, to insure a good electrical contact, the conductive buffer contained in the stainless steel cylinder was continuously refreshed by applying a small pressure (1psi) on the vial containing the conductive buffer. This resulted in a consistently stable spray and current for all runs, whatever their duration.

2.5 nano-LC

Reverse phase separation of peptides from milk digest was performed on a Ultimate 3000 LC RSLC nano-LC system (Dionex, Sunnyvale, CA). Sample was injected onto a C18 trapping column (Acclaim PepMap100: 100 μm×2 cm, 5 μm, 100 Å, Dionex). After 2 min washing with 2.0% MeCN, 0.1% FA at 300 nL/min, following valve switching, the sample was separated on a C18 nano column (Acclaim PepMap RSLC 75 μm×15 cm nanoViper, 2 μm 100 Å, Dionex) by a piece-linear gradient (5-40 min – 2-10%; 40-78 min – 10-30%; 78-83 min – 30-70%; 83-85 min – 70-90%; 85-90 min – 90% of mobile phase B, where B was 95% MeCN, 0.1% FA) at a constant flow rate 300 nL/min.

2.6 Mass Spectrometry

All initial infusion and method optimization experiments were performed on a MaXis 4G UHR-TOF mass spectrometer from Bruker Daltonics (Bremen, Germany) at 1 Hz

acquisition frequency. All tandem MS experiments were performed on an amaZon speed ETD ion trap instrument from Bruker Daltonics. MS scan was within a range of 300–1500 m/z with ion focus on 800 m/z. The ten most abundant multiple charged ions of an MS spectrum were selected for MS/MS analysis by collision-induced dissociation using helium as the collision gas with a precursor threshold of 10000 counts. In case of observation of a neutral loss fragment (26.7; 32.7; 38.6; 40.0; 49.0; 58.0; 65.0; 80.0; 98.0 Da) the multistage activation of this fragment was automatically triggered (fragment only mode). The masses corresponding to the fragmented ions were dynamically excluded for 0.1 min from further MSn analysis in the nano-LC-MS method. No exclusion was applied in the CE-MS strategy due to strongly varying peakwidths throughout the separation.

For the coupling of the sheathless CE sprayer to the mass spectrometer, a specially designed sprayer mount in combination with the Bruker nano spray shield was used. Generally, stable spray for positive ionization was achieved between -750 and -1500 V ESI Voltage, which was dependent on the distance between the sprayer tip and the MS entrance. Drying gas was set to 2 L/min (nitrogen) while the source temperature was set to 180 °C.

The coupling of the nano-LC to the ion trap mass spectrometer was performed via a Bruker CaptiveSpray ionization (CSI) source (Bremen, Germany). Stable spray for positive ionization was obtained at -1300 V ESI Voltage with the source temperature at 150 °C and the drying gas (nitrogen) set to 3 L/min.

2.7 Viscosity and flow rate measurement.

Generally, ESI voltage may cause an ultra-low flow in bare fused capillaries due to the combination of ESI suction and EOF in the spray tip during infusion experiments. Therefore, the flow rates at ultra-low pressures needed to be accurately determined for the infusion experiments performed in bare fused capillaries. A 30 µm i.d. × 150 µm o.d. × 100 cm bare fused capillary with a porous spray tip was filled hydrodynamically with BGE. The non-phosphorylated synthetic peptide dissolved in BGE was then continuously introduced hydrodynamically into the capillary while the ESI voltage was applied to the sprayer until the peptide was detected by the mass spectrometer. The absolute flow rate was determined by the capillary volume divided by the mobilization time. As some diffusion takes place the mobilization time was taken to be the time at half height between the moment the peptide was detected and the maximum signal intensity.

The combined ESI suction and EOF effect reduced exponentially at increased pressure; therefore the effect became negligible above 10 nL/min flow rates (1.4 psi pressure for infusion experiments). All higher flow rates were calculated from the BGE viscosity, which was measured with a previously reported method[29]. Briefly, in a CE-UV configuration, the capillary was filled with the studied BGE. Subsequently a short water plug (< 1% of total capillary volume) was hydrodynamically introduced in the capillary. The zone was then mobilized hydrodynamically until detection of the water plug by UV. Viscosity (η) was calculated from the mobilization time applying the Hagen- Poiseuille law:

$$\eta = dc2\Delta Pt/(32L2) \quad (1)$$

Where dc is the internal capillary diameter, ΔP the mobilization pressure, t the mobilization time, and L the length of the capillary. The flow rates were then calculated from the Poiseuille equation (2) taking the applied pressure and the experimentally determined BGE viscosity into account.

$$V = (\pi/128)dc4((\Delta Pt)/(\eta L)) \quad (2)$$

2.8 Data analysis

Peak lists were generated from the raw spectra files using ESI Compass for amaZon 1.3 Data Analysis V4 SP4 (Bruker Daltonics, Bremen, Germany) with an autoMSn method allowing 2000 compounds with an intensity threshold of 1000 counts and a 0.5 min retention time window, and exported as Mascot Generic Files (MGF). These files were searched against the bovine protein database (containing 27254 records) using the Mascot search algorithm (Matrix Science). The parameters of the search were: fixed modifications – methylthio(C); variable modifications – oxidation(M), phospho (STY); trypsin missed cleavages – 2; MS tolerance (with # 13C=1) - 0.5 Da; MS/MS tolerance - 0.5 Da.

3 Results and Discussion

3.1 Characterization of ionization behavior of phosphopeptides at ultra-low flow rates

In phosphoproteomics the frequency of incidence of protein phosphorylation defines the phosphopeptide concentrations in a conventional protein digest. Moreover, playing a key regulatory function in intracellular signaling these phosphopeptides are short lived and far from abundant. Consequently, the presence of very high concentrations of non-phosphorylated peptides can hamper phosphopeptide analysis due to co-elution or co-migration and subsequent ion-suppression under ESI conditions. Although the obvious solution for the reduction of ion-suppression of phosphopeptides is improved separation power, the generally low ionization efficiency of multiply phosphorylated peptides, even under non-suppressed conditions, is problematic. In previous investigations of the ESI process,[13, 14, 26, 30] it was shown that the application of ultra-low flow rates in nano-ESI can have significant advantages with regard to ion suppression effects and ionization efficiencies. For this reason it was important to investigate the effects of strongly reduced flow (<30 nL/min) on the ionization efficiency of peptides with extensively varying phosphorylation states.

To this end, a synthetic peptide containing four tyrosine moieties for the incorporation of zero to four phosphorylations was designed to serve as a model in a low flow infusion study of the phosphopeptide ionization process. The C-terminal amino acid was chosen to be an arginine to emulate the characteristics of peptides resulting from tryptic digestion, which is currently the most commonly used protein digestion strategy. These peptides were dissolved as a mix in BGE (10% acetic acid) in equimolar concentrations before direct hydrodynamically driven infusion. An initial infusion experiment showed a clear change in signal profile between infusion at high flow rates (>100 nL/min) and low flow rate (~10 nL/min) (Figure 1-1) warranting further investigation.

To track the changes in relative ionization for the model peptides, an infusion experiment was designed and performed as follows. After hydrodynamic filling of the capillary the pressure was varied from 0.2 psi to 60 psi in steps of 0.2 to 10 psi with continuous MS detection. At each tested flowrate, the ESI voltage was optimized for spray stability and for the highest summed signal intensity of the [M+2H]2+ and [M+3H]3+ ions of the non-phosphorylated model peptide. Although each pressure was applied for two minutes, only 1.8 minute intervals were integrated for data analysis, allowing 0.2 minutes interval

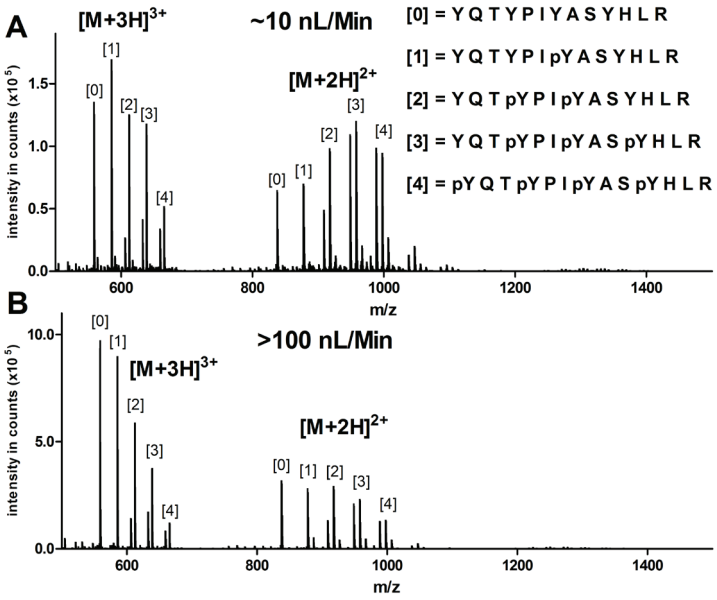

Figure 1-1: Typical mass spectra of the model phosphopeptide mix infusion at flows below 10 nL/min and above 100 nL/min. Only double and triple charged species are observed and the peaks are labeled with a number corresponding to their number of phosphorylations. Additional masses can be observed corresponding to a -18 Da mass difference from the di- to tetra-phospho form. These masses are due to impurities after synthesis.

after pressure increase for spray stabilization. The peak intensities of the (M+2H)2+ and (M+3H)3+ were summed and thus reported as the total signal at each flow rate for each respective peptide. For all 5 infused peptides a stable signal could be observed within the two minute windows. Investigating the signal intensities at flow rates below 100 nL/min (Figure 1-2A), it could be discerned that the evolution of the signal intensity as a function of the flow rate was not equal for all phosphopeptides. To better assess differences in behavior for each phosphopeptide present in the mixture, the ratio of its intensity with respect to the non-phosphorylated was plotted (Figure 1-2B). The ratio plot clearly shows that the ionization bias observed at conventional flow rates (> 50 nL/min) is significantly reduced at ultra-low flow rates. If only the most extreme case corresponding to the tetra-phosphorylated peptide is considered, it can be observed that its intensity only corresponds to less than 20% of the one of the non-phosphorylated peptide at flow rates above 50 nL/min while it accounts for more than 60% below 10 nL/min. Keeping in mind the work of Busnel et al.[26] and Marginean et al. [14] who studied ionization efficiency at ultra-low flow rates, it was shown that the detection sensitivity for all phosphorylated peptides is strongly enhanced when working at flow rates below 30 nL/min. The respective increase is dependent on the number of phosphorylation

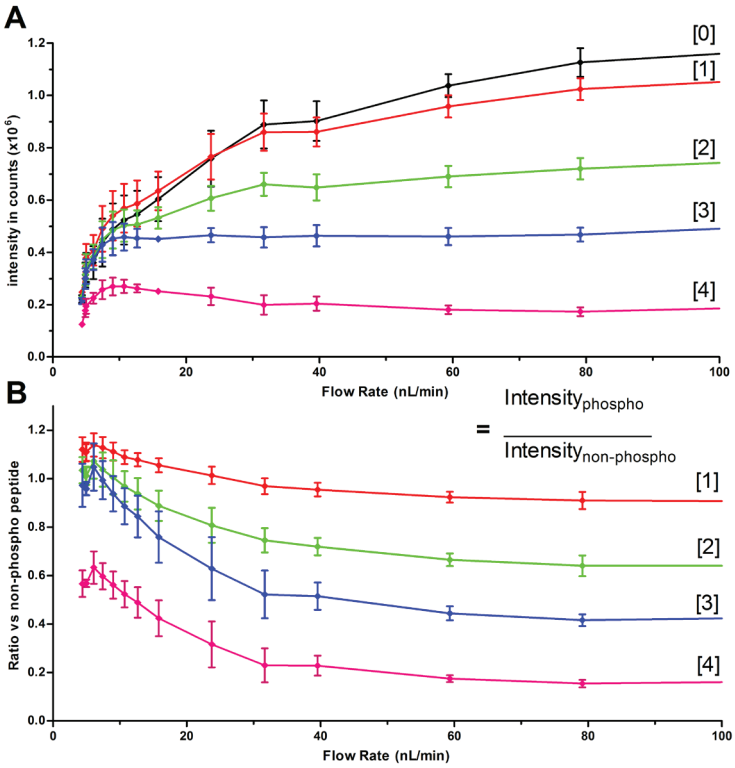

Figure 1-2: (A) Evolution of peak intensity of model phosphopeptides below 100 nL/min; (B) Signal intensity ratio as a function of the non-phosphorylated peptide. Experimental conditions: Bare fused silica capillary with porous tip, total length 30 µm i.d. × 150 µm o.d. × 100 cm; infusion of phosphopeptide mix at equimolar 5 µM concentration in 10% acetic acid. Mass spectrometry; capillary voltage was optimized for each flowrate (-1050 to -1275 V); detection range 300-2900 m/z, other experimental conditions described in Materials and Methods. (Black) non-phosphorylated [0], (Red) mono-phosphorylated [1], (green) di-phosphorylated [2], (Blue) tri-phosphorylated [3], (Pink) tetra-phosphorylated [4]

sites and therefore the largest increase in relative sensitivity was found for the tetra-phosphopeptide (Factor 4). This confirms, as previously shown by Schmidt et al. [13], the existence of an ESI regime (flow rate region below 30 nL/min) where common ionization bias can be reduced to such a large extent that the MS detection shows a trend toward an equimolar response[31]. Although the peptides were infused in equal concentration, the signal intensity of the tetra-phosporylated peptide did not equal that of the other four model peptides. Nevertheless, it experienced the greatest increase in relative signal intensity.

After having assessed with an infusion-based approach the potential impact of lowering the flow rate on the ionization efficiency of different phosphopeptides, the next step was to understand to which extent we could take advantage of this behavior in an ultra-low flow separation system.

3.2 Neutral capillary coating in sheathless tITP-ESI-MS

3.2.1 Optimization of analyte stacking

Our infusion experiments demonstrated phenomena, which are of particular importance for the analysis of phosphopeptides. To improve total peptide coverage in a complex sample, however, an analytical strategy which combines ESI under ultra-low flow with an efficient separation technique has to be found. Regrettably, there are just a few separation techniques which are compatible with the flow rates required to achieve close-to-equimolar ionization (<10 nL/min). Although nano-splitting has been applied in some studies to achieve these very low flow rates in liquid chromatography[32], this is a far from common practice as it can easily result in band broadening and additional/unwanted dead volumes. Porous layer open tubular (PLOT) columns, for example, can efficiently operate at flows rates of a similar order of magnitude (~20 nL/min)[33]. Currently, these columns require in-house manufacturing as they are not commercially available, and their operation is challenging because of their extreme dimensions (10 µm ID and up to several meters long) and the nano splitting pumps required to produce these flowrates. Only CE separation using neutrally coated capillaries aided by hydrodynamic pressure produces a straightforward approach to achieve excellent separation while maintaining consistent flows below 15 nL/min.

When implementing CE in neutrally coated capillaries as the separation strategy, as opposed to the use of bare fused capillaries, no EOF is produced and the flow required for electrospray has to be produced by the application of a slight hydrodynamic pressure at the capillary inlet. Although it has now been shown that ultra-low flow ESI can result in a significant increase of sensitivity, CE, as compared to LC based techniques, has a significant limitation with regard to loadability. Additionally, as phosphorylations and therefore phosphopeptides are generally only present in complex samples at ultra-low concentrations, the use of larger volume injections would be greatly beneficial. As a rule of thumb, in traditional CZE separations, the sample plug is usually limited to about 1 % of the total capillary volume because larger sample volumes result in broad peaks and subsequently reduced resolution. To increase loadability in CE while maintaining high efficiency capabilities, transient-isotachophoresis (t-ITP) [34] and other stacking strategies have been developed. Significant increases of sensitivity can be achieved as sample volumes up to 50% of the total capillary volume can be injected while maintaining high efficiency and satisfactory resolution. Depending on the volume of the used separation capillary, varying degrees of analytical resolution and loadabilities can be achieved.

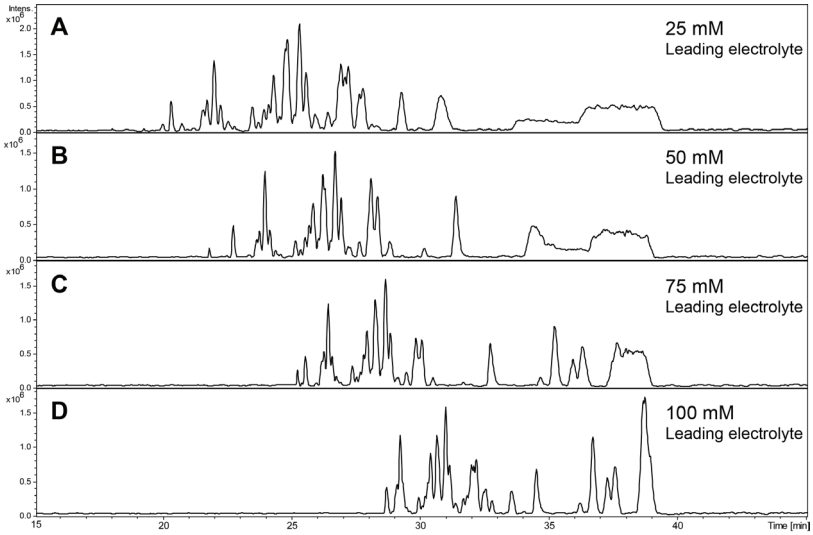

Figure 1-3: Neutral capillary t-ITP-MS analysis of 2,5 ng milk digest (37% capillary fill, 30 psi for 60 s) Base Peak electropherogram. Mass spectrometry; ESI voltage of -1050V; detection range 300-2900 m/z, other experimental conditions described in Materials and Methods (A) sample dissolved in 25 mM leading electrolyte (LE) (B) sample dissolved in 50 mM leading electrolyte (C) sample dissolved in 75 mM leading electrolyte (D) sample dissolved in 100 mM leading electrolyte

Unfortunately, the use of an ITP strategy can result in great losses in resolution. Firstly, the capillary length used to achieve electrophoretic resolution is reduced as a significant portion of the capillary is used to load the sample of interest. Secondly, the composition and concentration of leading electrolyte (LE) has a great influence on the level of stacking and the degree of resolving power in an ITP strategy. As phosphopeptides generally have a low isoelectric point due to their highly acidic phosphate moieties, it could be expected that high concentrations of LE are required for optimal stacking. To evaluate the required concentration of LE in our ultra-low-flow sheathless t-ITP-CE-ESI-MS system, a digest of bovine milk was taken as a model sample. Bovine milk is a biological sample of medium complexity with a large proportion of the protein content corresponding to caseins. These 4 caseins (αS1, αS2, β and κ) have abundant phosphorylations and make bovine milk an ideal model sample to optimize phosphoproteomic workflows.

To achieve a significant loading capacity, the sample volume was set to 250 nL (37% of the total capillary volume). Figure 1-3 shows the varying levels of stacking and of analytical resolution achieved in the scope of the optimization of the stacking conditions. LE ionic strengths were varied from 25 mM to 100 mM while separation was performed with 0.8 psi of pressure applied at the inlet to hydrodynamically induce flow in the capillary to achieve a stable ESI spray. This hydrodynamic pressure was determined empirically during the infusion experiments. A separation voltage of 25 kV was chosen to achieve

optimal separation while maintaining a separation current below 6 µA. As determined by using the last peak in the electropherogram as a marker, complete stacking was achieved at 100 mM ionic strength (Figure 1-3D). The effective separation window was reduced to only 10 minutes as a result of the high concentration of LE and the short capillary length (63%) left for actual preconcentration/separation. Although the reduced resolution is not detrimental to the ionization efficiency as the low flow ESI process has shown minimal ionization suppression for phosphopeptides, the mass spectrometric analysis of complex samples could be significantly hampered at low analytical resolution as tandem mass spectrometry of many co-eluting compounds is difficult, mainly due to technical limitations in the number of MSn events per time unit.

3.2.2 Tandem mass spectrometry identification of phosphopeptides with t-ITP separation followed by sheatless-CZE-MS

The above described separation strategy was applied to analyze the bovine milk digest sample. To further assess the potential of the ultra-low flow CE-ESI-MS method for the analysis of phosphopeptides, 5 ng of milk digest was loaded on both CE and nano-LC systems and the results obtained from a merge of 4 technical replicates with both approaches were compared. The mass spectrometric method was optimized for phosphoproteomic fragmentation which included the secondary fragmentation of any neutral loss ion related to a phosphopeptide. The fragmentation of this neutral loss compound does not result in a full MS3 spectrum but in a cumulative MS2 plus MS3 spectrum. As both CE and LC do not appear to provide resolution for peptides with the same peptide backbone but with varying phosphorylated sites, peptides identified by the MASCOT search with equal backbones but varying phosphorylatied sites were assigned as one unique peptide. Table 1-1 clearly shows the advantages of the CE-MS phosphoproteomic strategy with regard to minute sample amounts as 13 phosphopeptides could be determined, including di- phosphorylated peptides while only 5 phosphorylated peptides were identified by the nano-LC based approach.

Although the absolute sensitivity of the CE-MS strategy is undeniable, an argument can be made for the capability of the nano-LC system to load larger amounts of sample onto the system. In this context, a larger amount of material (50 ng) was additionally loaded on the nano-LC-MS system (2.5 µL of a sample at 20 ng/µL). The merged data of 4 technical replicates of this significantly higher sample load only yielded four additional phosphopeptides as compared to the 5 ng sample load, while not resulting in detection of multiply phosphorylated species. Although only three phosphopeptides detected by

analysis characteristics	no. of phosphopeptides	1P	2P	unique phosphopeptides
5 ng of milk digest tITP–MS	12	10	2	5
5 ng of milk digest nano-LC–MS	5	5	0	1
50 ng of milk digest nano-LC–MS	9	9	0	3

Table 1-1: Comparison of the detected phosphopeptides from a bovine milk digest. The t-ITP-MS method consisted of 0.8 psi pressure for 60 min (6.7 nL/min) with 25 kV separation and 1100 V ESI voltage. The nano-LC−MS method consisted of a 300 nL/min water/acetonitrile gradient for 90 min. Other experimental conditions are described in the Materials and Methods. The peptide numbers are a result of a merge of multiple analyses of the same sample (n = 4). Only phosphopeptides with a peptide score above 25 were included in the table.

nano-LC-MS were unique for their separation technique, it does indicate some form of complementarity to the two techniques.

The difference in phosphopeptide detection between CE-MS and nano-LC-MS can certainly be explained by an accumulation of multiple factors. Naturally, the previously discussed increase in ionization efficiency at low flowrates is of great influence. Secondly, the nature of the separation strategy could be a contributing factor as number of the detected peptides are highly polar and therefore interact only poorly with the used stationary phases. Subsequently, it is indeed possible that a portion of the peptides detected by CE-MS were not retained on the trap-column before the chromatographic separation. As the CE-MS strategy did not contain a trapping protocol, all compounds in the sample had indeed the potential to reach the detector.

Reversely, while the ionization process in sheathless CE-ESI-MS at ultra-low flow rates is very favorable to the MS detection of multi-phosphorylated peptides, it is also very likely that a number of phosphopeptides remain undetermined due to the intrinsic nature of the CE separation mechanism. As highly phosphorylated peptides can indeed present a ultra-low pI, they can be negatively charged, even at pH 2.2, and therefore migrate toward the inlet of the capillary rather than toward the MS. As a consequence, it is likely that a portion of the phosphoproteome was missed by the considered CE-based analytical strategy. Consequently, a complementary CE strategy to the one presented here will have to be developed to achieve comprehensive phosphoproteomic analysis, if CE is to be used as the standard method in future investigations.

4 Conclusions

In this study, the potential of ultra-low flow CE-ESI-MS was investigated for phosphoproteomic analysis. The influence of the ESI flow rate on the ionization efficiency of a number of synthetic peptides showed a significant increase in phosphopeptide ionization at ultra-low flow rates. The flowrate could be decreased to such an extent that a near equimolar ESI response was approached for flowrates below 15 nL/min.

Applying the knowledge of ultra-low flow ESI and combining it with CE capabilities, a sheathless t-ITP-CE-ESI-MS strategy was developed using an ammonium acetate buffer at pH 4.0 as a leading electrolyte. Although the nature of the stacking process reduces the electrophoretic resolution compared to conventional CZE, unprecedented sensitivities could be achieved in the detection of phosphopeptides. When compared to nano-LC-MS, the proposed strategy was superior in both absolute (equal sample amount loaded) and concentration sensitivity (equal sample concentration). The developed CE-MS strategy was able to identify 2 multi-phosphorylated peptides from the sample whereas the nano-LC-MS was only able to identify mono-phosphorylated peptides, even in the high sample load analysis. Moreover, the use of ultra-low flow ionization also shows improved ionization of pSerine peptides as the doubly phosphorylated peptides (supplementary information) were both pSerine peptides, the pSer and pThr moieties are more labile than the investigated pTyr. Therefore, the stability of pSer and pThr at ultra-low flow ionization need to be investigated when considering it as a strategy in quantitative phosphoproteomics.

In conclusion it was shown that ultra-low flow ESI greatly increases the detection sensitivity for multi-phosphorylated peptides in mass spectrometric analysis and that this feature can be translated into a sheathless CE-ESI-MS platforms strategy. Although a sheathless CE-ESI-MS strategy was applied to achieve separation at the required flowrates (<10 nL/min), in principle any liquid based separation strategy (also LC or CEC) could be applied in concert with ultra-low flow ESI. As Schmidt et al.[13] have shown that the observed effect was also present for a 50% methanol solution it can be concluded that ultra-low flow ESI should also be greatly beneficial to conventional RP-LC-MS strategies using organic modifier based separation.

As this report was a proof of principle investigation for mass spectrometric analysis in phosphoproteomics, no sample pretreatment and preconcentration techniques were investigated for further improvement of phosphoproteomics analysis. Therefore, ultra-

low flow LC separation techniques sample pretreatment techniques that are compatible with the applied CE-MS strategy should be the target of future investigation for improved phosphoproteomic work flows.

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Chapter 2

Coupling porous sheathless interface MS with transient-ITP in neutral capillaries for improved sensitivity in glycopeptide analysis

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Abstract

Immunoglobulin G antibodies are modulated in their function by the specific structure of the N-glycans attached to their Fc portions. However, the glycosylation analysis of antigen-specific IgGs is a challenging task as antibody levels to a given antigen only represent a fraction of the total IgG levels. Here, we investigated the use of a transient-isotachophoresis (t-ITP) - mass spectrometry method for highly sensitive immunoglobulin G1 (IgG1) glycosylation profiling as a complementary method to a high throughput nano-reverse phase liquid chromatography-mass spectrometry (nano-RPLC-MS) method. It was found that t-ITP-CZE using neutrally coated separation capillaries with a large volume injection (37% of capillary volume) and interfaced to mass spectrometry with a sheathless porous sprayer yielded a 40 fold increase in sensitivity for IgG1 Fc glycopeptide analysis when compared to the conventional strategy. Furthermore, the glycoform profiles found with the t-ITP-CZE strategy were comparable to those from nano-RPLC-MS. In conclusion, the use of the highly sensitive t-ITP-CZE-MS method will provide information on IgG Fc glycosylation for those samples with IgG1 concentrations below the limits of detection of the conventional method.

1 Introduction

Human immunoglobulin G (IgG) molecules contain two heavy chains and two light chains forming together 2 Fab (fragment antigen binding) portions and 1 Fc (fragment crystallizable) portion. Four subclasses of human IgG exist (IgG1, IgG2, IgG3 and IgG4) named in the order of decreasing concentration in human blood [1]. The Fc portions of human IgG contain a conserved N-glycosylation site at Asn 297 of the CH2 domains of their heavy chain. N-glycosylation is important for modulation of IgG activity and is known to be dependent on age, gender as well as on physiological and pathological conditions of an organism [2-9].

Consequently, the analysis of Fc glycosylation of antigen-specific affinity-purified IgGs provides insight into the (dys-)regulation of IgG glycoyslation features in health and diseases. For example, we have recently shown that IgG1 directed against human vaccines shows a transient increase of galactosylation and sialylation within the first couple of months after vaccination [10]. In rheumatoid arthritis, pathogenic anti-citrullinated protein antibodies isolated from the inflamed joint of rheumatoid arthritis patients exhibited low galactosylation and sialylation which is interpreted as a pro-inflammatory glycosylation profile [11]. Finally, it has been shown that low core-fucosylation is a characteristic feature of pathogenic IgG1 allo-antibodies produced by pregnant women against platelet antigens of the fetus [5]. These antibodies are transported via the placenta and can destroy the fetus' platelets, which results in petechiae, major organ bleedings or even intracranial hemorrhages (Fetal/neonatal allo-immune thrombocytopenia; FNAIT). Thus, low core-fucosylation may represent an important pathogenic factor promoting platelet destruction as it is known to lead to enhance ADCC [12].

A number of analytical techniques have been developed for the analysis of protein glycosylation in general, and for the characterization of IgG glycosylation in particular. Commonly used strategies for mass spectrometric IgG glycosylation profiling in proteolytic digests are reversed phase and hydrophilic interaction liquid chromatography coupled to mass spectrometry (RPLC-MS and HILIC-MS) and direct mass spectrometric analysis of glycopeptides by MALDI-MS [13, 14]. In recent years capillary electrophoresis mass spectrometry (CE-MS) has been applied for the analysis of glycopeptides from not only IgG but also erythropoietin proteolytic digests [13-16].

We have developed a robust, high-throughput nano-RPLC-MS method for IgG N-glycosylation profiling [6]: human polyclonal IgGs are captured from 2 µl of plasma or serum by protein G-SepharoseTM beads (GE Healthcare, Uppsala, Sweden) and cleaved overnight with 200 ng of trypsin [17], followed by nano-LCTOFMS analysis of glycopeptides with a lower limit of detection of approximately 870 attomol [6].

The sensitivity of the currently available glycoanalytical methods [6, 13] are found to be sufficient for detailed analysis of total IgG Fc glycosylation from sub-micro liter volumes of plasma or serum. These same methods, however, fall short if applied for the analysis of antigen-specific IgG, allowing the analysis of patient samples with high antigen-specific IgG titers only. Using larger serum or plasma volumes to be able to capture sufficient amounts of the pathogenic auto- or allo-antibodies is not always possible due to limited sample amounts that are available for a single assay as multiple assays are likely required from the same precious sample.

Therefore, we decided to set up a highly sensitive IgG Fc glycosylation assay for the analysis of tryptic Fc glycopeptides on the basis of a previously characterized transient-isotachophoresis (t-ITP) separation strategy. This strategy performs electrophoretic separation in neutrally coated capillaries with porous sheathless interfacing to a mass spectrometer[18, 19]. Similar analytical strategies have previously provided significant improvements in proteomic analysis of various samples [20, 21].

2 Experimental

All analyses were carried out using a Beckman Coulter PA800 plus (Brea, California USA) coupled to a MaXis 4G UHR-TOF from Bruker Daltonics (Bremen, Germany) via a porous sheathless interface designed by Mehdi Moini [18], and now in development by Beckman Coulter. The mass spectrometer was tuned with highest sensitivity in the m/z 700 to 1600 region to register all IgG derived tryptic Fc glycopeptides. The mass spectrometer was operated in positive ionization mode and acquired data in the mass range from m/z 400 to 2900 with a spectra rate of 1 Hz. The permanent capillary coating, currently in development by Beckman Coulter, consisted of a bi-layer with the outer surface based on polyacrylamide. The background electrolyte consisted of 10% acetic acid and the sample buffer/leading electrolyte of 100 mM ammonium acetate pH 4. 37% of the total capillary volume was filled with the sample and 25 kV was applied across the capillary for separation. Concomitantly to the voltage application, a pressure of 1 psi was applied at the capillary inlet to obtain a flow sufficient for stable electrospray

(approximately 8 nl/min). All chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated. Every sample run was internally calibrated using polysiloxane clusters which were present in the mass spectra due to the open source design and the nanoflow regime.

3 Results and Discussion

A tryptic digest of total human plasma IgG purified by protein-G was analyzed, and in accordance with previous observations based on analysis by nano-RPLC-MS [6] 18 IgG1 glycoforms could be determined as both [M+2H]2+ and [M+3H]3+. Structural assignment of the detected glycoforms was performed on the basis of literature knowledge of IgG N-glycosylation [22-26]. For the considered glycopeptides, the t-ITP-CZE system did not show a separation on the basis of the peptide moieties but rather on basis of the attached glycan (Figure 2-1), while glycopeptides from the different IgG subclasses were found to co-migrate (data not shown). This is in contrast to nano-RPLC-MS where a complete separation of the three subclass-specific glycopeptide clusters was observed on the basis of minor differences in peptide sequence [6].

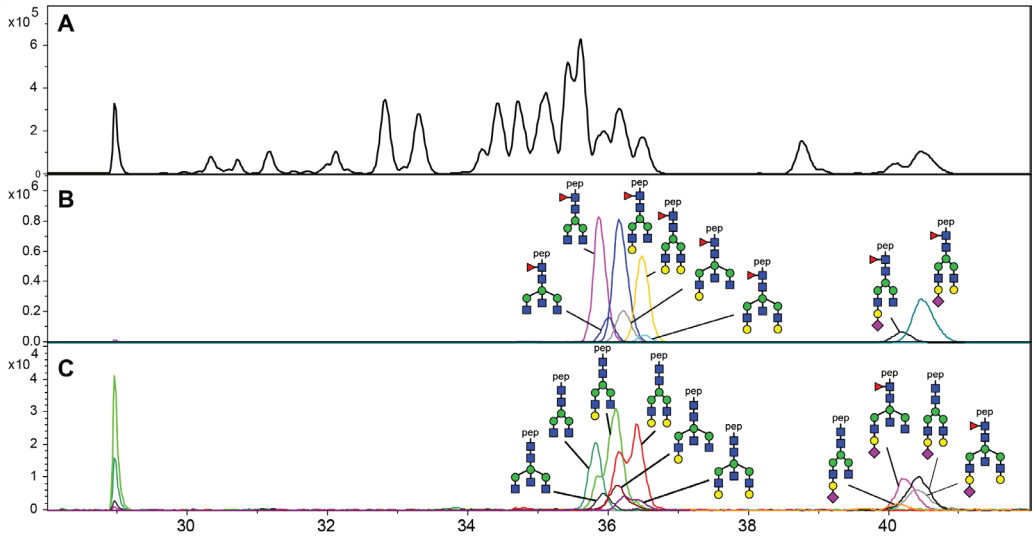

Figure 2-1: (A) Base peak electropherogram of a tryptic digest of total IgG purified from human serum; (B) extracted ion traces of the high concentration IgG1 glycopeptide species including assignment; (C) extracted ion traces of the low concentration IgG1 glycopeptide species including assignment. Blue square, N-acetylglucosamine; red triangle, fucose; green circle, mannose; yellow circle, galactose; purple diamond, N-acetylneuraminic acid.

A comparison of the nano-RPLC-MS [6] and t-ITP-CZE-MS methods was performed using 250 nl of the protein G purified plasma IgG digest. The comparison was performed using a total IgG digest opposed to real patient samples as the samples were only obtained in very low quantities which were insufficient to perform the appropriate comparisons.The IgG digest was used undiluted for nano-RPLC-MS but was diluted a factor 20 for the t-ITP-CZE-MS method to prevent detector saturation. Normalized intensities of IgG1 glycoforms were determined by scaling the peak area of each peak to the total obtained peak area for all detectable IgG1 glycoforms. It was found that the relative abundances of the major glycopeptides were very similar for the two methods (Table 2-1). Subsequently, the sample was analyzed on 3 consecutive days to determine the inter-day reproducibility of the t-ITP-CZE-MS method for larger studies. The inter-day reproducibility in the t-ITP-CZE-MS method showed to be less than that observed for the nano-RPLC-MS (Table 2-1). In view of the fact that this technique allows a significant increase in sensitivity for glycosylation analysis, the observed inter-day reproducibility is certainly acceptable.

Glycan code[a)]	Compound (m/z [b)])		t-ITP-CZE-MS			RPLC-MS		
	$[M+2H]^{2+}$	$[M+3H]^{3+}$	Relative intensity normalized to 100[c)]	SD	RSD	Relative intensity normalized to 100[c)]	SD	RSD
G0F	1317.527	878.687	23.60	1.54	6.53	21.94	0.35	1.61
G1F	1398.553	932.705	28.06	1.32	4.70	29.73	0.18	0.62
G2F	1479.58	986.722	17.31	0.29	1.67	16.91	0.18	1.07
G0FN	1419.067	946.38	5.17	0.35	6.83	5.50	0.19	3.52
G1FN	1500.093	1000.398	7.41	0.42	5.60	7.72	0.14	1.82
G2FN	1581.119	1054.416	1.50	0.02	1.64	1.50	0.09	6.09
G1FS	1544.101	1029.737	2.62	0.27	10.50	2.39	0.08	3.34
G2FS	1625.127	1083.754	11.62	2.03	17.49	10.26	0.41	3.97
G1FNS	1645.641	1097.43	0.39	0.03	7.04	0.62	0.03	5.39
G2FNS	1726.667	1151.447	0.30	0.04	12.19	0.38	0.04	11.77
G0	1244.498	830.001	0.61	0.04	6.29	0.90	0.05	5.01
G1[d)]	1325.524	884.019	–	–	–	3.06	0.27	8.68
G2[d)]	1406.551	938.036	–	–	–	3.35	0.13	3.99
G0N	1346.038	897.694	0.19	0.02	8.58	0.26	0.06	23.14
G1N	1427.064	951.712	0.39	0.10	25.42	0.45	0.12	25.95
G2N	1508.09	1005.73	0.30	0.13	43.13	0.48	0.10	20.79
G1S	1471.072	981.051	0.06	0.01	13.52	0.31	0.04	12.48
G2S	1552.098	1035.068	0.48	0.02	4.15	0.65	0.05	7.45

Table 2-1: Comparative analysis of a standard IgG1 glycopeptide samples by t-ITP-CZE-MS and RPLC-MS. SD, standard deviation; RSD, relative standard deviation

a *Calculated monoisotopic m/z values of the [M+2H]2+ and [M+3H]3+ species are given.*
b Glycan compositions are given in terms of number of galactoses (G0, G1, G2) and presence of fucose (F), bisecting N-acetylglucosamine (N), and sialic acid (S).
c The areas of the first, second and third isotopic peaks of the double protonated and triple protonated species were summed.
d Glycopeptides were excluded because of co-migrating contaminant in CE separation only present in the standard but not in patient samples.

The limits of detection and the dynamic range of the t-ITP-CZE-MS method were determined as described previously [6]. Briefly, 250 nl of two monoclonal antibody tryptic digest dilutions at 4, 16, 63, 250, 1000 and 4000 pg/µl were analyzed by a single analysis per concentration. In the Fc portion of each antibody two N-glycans are located, which may vary in glycan composition. Upon analysis of the lowest measurable concentration (16 pg/µl) three different glycoforms were observed in the analysis of both antibodies. The lower limit of detection (LOD) was estimated to be 20 amol for the individual glycopeptides which amounts to 80 pM sample concentration and was calculated by:

$$\frac{\text{Lowest measurable antibody concentration (16 pg/µl) * Number of Fc N-glycans (2)}}{\text{Molecular weight antibody (150 000) * Number of observed major glycoforms (3)}}$$

This is an improvement in sensitivity by a factor of 40 in the lower LOD for t-ITP-CZE-MS as compared to nano-RPLC-MS. The higher LOD (detector saturation) was not “challenged” as the high concentration samples are always analyzed by the RPLC-MS method [6]. Nevertheless, the detection was observed to be linear over at least 2 orders of magnitude from the low LOD with R2 for both dilutions series above 0.99 for the three observed glycans. Notably, the achieved sensitivity is also better than that of recently tested MALDI-TOF-MS methods which achieved an LOD of between 40 and 400 amol on the MALDI target spot, depending on the applied matrix, sample preparation method, and ionization mode [6]. With regard to the MALDI-TOF-MS methods, it has to be mentioned that the LOD was determined using dilution series of highly concentrated and pure IgG, thereby limiting the potential negative influence of salts on the ionization process. One has to take into account, however, that the reported sensitivity will most probably not be achieved on scarce biological samples, as those will require additional sample preparation/desalting steps with the concomitant risk of sample loss.

The t-ITP-CZE system was then applied to analyze the IgG Fc-glycosylation of anti-Human Platelet Antigen 1a (HPA1a) allo-antibodies purified from serum of pregnant women. Briefly, allo-antibodies were affinity-purified exploiting their binding to the HPA1a antigen and subsequently digested with trypsin [5]. After digestion samples were dried down and reconstituted in 2.5 µl of 100 mM ammonium acetate buffer which functions as a leading electrolyte. Analysis of 250 nl aliquots by t-ITP-CZE provided high-quality IgG1 Fc glycopeptide profiles. These IgG1 Fc glycopeptide profiles reveal vast differences in core-fucosylation and galactosylation as illustrated in Figure 2-2. Sample 1 showed a high degree of both core-fucosylation and galactosylation (integration of the data revealed

that 81% of glycans were fucosylated and 81% of the possible sites were galactosylated; Figure 2-2A) while sample 2 showed a very low degree of core-fucosylation and high degree of galactosylation (only 17% of all glycans were fucosylated while galactosylation was 83%; Figure 2-2B). Finally, sample 3 exhibited a high degree of core-fucosylation but low galactosylation (86% and 48% respectively; Figure 2-2C). This is consistent with previous observations by nano-RPLC-MS where we found heterogeneity of anti-HPA IgG1 allo-antibodies with regard to the degree of core-fucosylation and galactosylation [5]. As mentioned above, these differences in core-fucosylation and galactosylation may be functionally important as low core-fucosylation can vastly enhance the pathogenicity of allo-antibodies via increased ADCC activity and low galactosylation may be indicative of a pro-inflammatory response [5, 11, 12].

4 Conclusion

In this paper we present a t-ITP-CZE-MS method as an auxiliary method to our nano-RPLC-MS glycopeptides profiling workflow. The obtained glycosylation profiles are very similar to those determined using the recently described nano-RPLC-MS method. As the sample treatment to change from nano-RPLC-MS to t-ITP-CZE-MS is performed in a very straightforward manner by drying the sample followed by reconstitution in the required sample buffer,the two analytical strategies are complementary to each other. Therefore, sample that contains glycopeptide concentrations below the limit of detection for the nano-RPLC-MS method can be re-analyzed using the high sensitivity t-ITP-CZE-MS method and provide additional data without any extensive sample preparation or use of additional untreated sample. Alternatively, the samples may be stored for several months at -20oC after evaporation, making it possible to analyze the samples at a later time point.

The developed t-ITP-CZE-MS method is of limited value for the analysis of large sample sets due to the rather low throughput (60 min per sample) as compared to the previously developed nano-RPLC-MS method (16 min per sample). However, t-ITP-CZE-MS will serve as a complementary technique to analyze those samples with IgG levels below the current detection limits of the nano-RPLC-MS method.

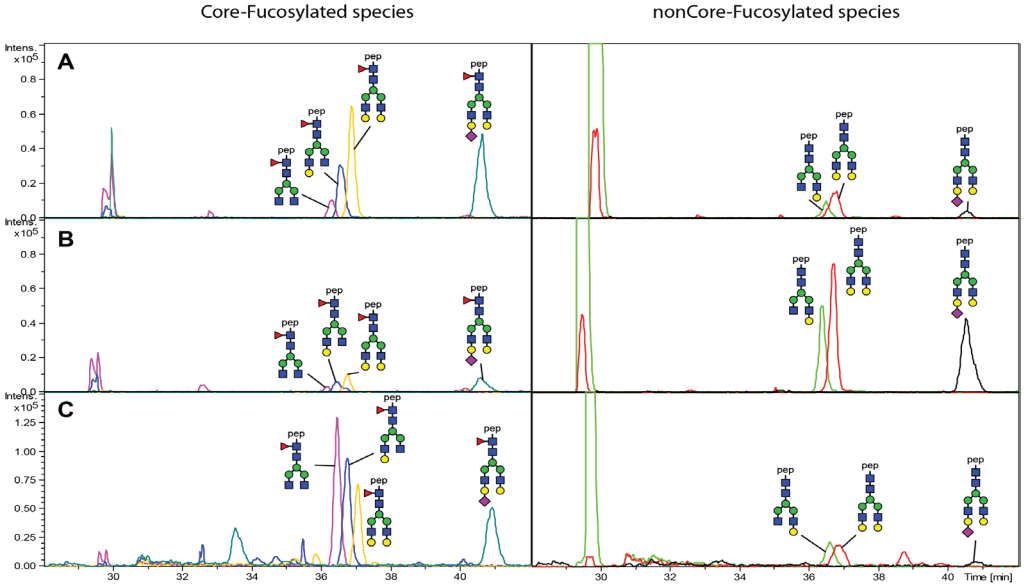

Figure 2-2: IgG Fc-glycosylation profiles of HPA1a alloantibodies purified from sera of three pregnant women. Extracted ion traces are given of the major core-fucosylated (left) and non-core-fucosylated (right) glycopeptide species. The first HPA1a glycosylation profile is high in fucosylation, galactosylation and sialylation (A), the second shows low fucosylation with high galactosylation and sialylation (B), and the third shows low galactosylation and sialylation with high fucosylation (C). Blue square, N-acetylglucosamine; red triangle, fucose; green circle, mannose; yellow circle, galactose; purple diamond, N-acetylneuraminic acid.

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Chapter 3

Optimization of capillary electrophoresis-mass spectrometry loadability and separation power

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Unpublished

Abstract

The potential of separation at stagnant solvent condition was investigated in large volume injection capillary electrophoresis. In previous investigations it was found that the use of large volume injection Transient Isotachophoresis (t-ITP) resulted in significant increases in loadability and sensitivity at the cost of a strong reduction in resolving power. In this investigation it was hypothesized that the application of separation voltage to a separation capillary with a neutral internal coating could result in separation conditions without a significant electro osmotic flow (EOF). This ‘Zero-flow’ separation should theoretically result in a significant improvement of the peak capacity when compared to separation under EOF or hydrodynamicly induced flow conditions. During the investigation it was found that the use of ‘Zero-Flow’ separation is possible in the sheathless CE-MS hyphenation setup, and that a 100% increase in peak capacity could be realized in a 34% capillary fill t-ITP-CZE strategy.

1 Introduction

Since the first reports of capillary gel electrophoresis[1] and free solution capillary zone electrophoresis (CZE),[2-4] electrokinetic based separation methods have received wide acceptance in biomedical and analytical sciences. With the explosive development of mass spectrometry, characteristics of capillary electrophoresis such as operation at low flow condition, low sample consumption and high separation power have commonly been recognized as advantages of the method. However, this separation power comes at a significant price, namely sensitivity, as the highest separation is achieved if the sample plug is kept to a minimum (1-2% of the volume of the capillary).[5] The use of isotachophoresis (ITP)[6] and electrokinetic supercharging[7] significantly increases the sample loadability, and consequently the sensitivity, of capillary electrophoretic techniques. Still, the use of these stacking techniques results in peak capacity power, especially in the case of ITP where a significant portion of the separation capillary is filled which reduces the effective capillary length. Moreover, the leading electrolyte required for transient-ITP stacking reduces the separation window even further as it forms a highly conductive zone which reduces the velocity of the analytes resulting in stacking before separation occurs.[5]

Control of electro-osmotic flow (EOF) is one of the possible mechanisms for tuning the separation power. CE (unlike LC) requires little to no linear flow and the EOF can therefore be reduced by capillary coatings based on modifying (neutralizing) the surface charge of the inner capillary wall.[8] The strong reduction of the EOF to negligible levels results in an improved electrophoretic mobility to EOF ratio, resulting in higher resolution. In ITP-CZE-UV or ITP-CZE-LIF separation the use of this operation at Zero-flow or even reversed flow results in optimal use of the remaining capillary length after large volume injections, despite the large leading electrolyte plug. Unfortunately, the various strategies[9-11] that have been developed for CE-MS hyphenation have strongly reduced the use of neutrally coated separation capillaries as the majority of themrequire a significant flow to obtain a closed circuit. [12, 13] The approaches that don’t require a flow in the separation system on the other hand require the use of some form of a sheathliquid or liquid junction interface.[14, 15]

Recently, Busnel et al. showed the use of a porous-tip sheathless interface as described by Moini[16] for the CZE separation of protein digests before ESI-MS analysis.[17] In their investigation the use of large volume injection through t-ITP in combination with very low flow separation was evaluated. They found that using neutrally coated capillaries,

separation could be achieved at flow rates of only 4.5 nl/min resulting in a peak capacity of 320 while using t-ITP injections. As the flow through the analytical system was almost negligible this begged the question whether any flow was actually required to maintain a closed circuit. If no flow would be required, the full potential of CE separation power could be used by not applying a pressure to the separation capillary until the analytes are close to the outlet of the capillary. This would then provide high CE separation power while increasing the loadability through t-ITP.

2 Materials and Methods

2.1 Chemicals

All used chemicals and standards were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and of analytical reagent grade. Powdered bovine milk was purchased locally. Nano-pure water from an Alpha-Q Millipore (Amsterdam, The Netherlands) was used for all buffers and solutions.

2.2 Sample preparation

Dry milk powder (20 mg) was resuspended in 5 mL 50 mM TEAB (triethylammonium bicarbonate buffer, pH 8.0, Fluka) containing 0.1% of RapiGest (Waters, Milford, MA). The cysteines were reduced with 2 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride) for 45 min at 60 °C and subsequently alkylated with 4 mM MMTS (S-methyl methanethiosulfonate, Fluka) for 30 min at room temperature. Proteins were then digested with trypsin overnight at 37 °C (Sequencing grade modified trypsin, Promega, Madison, WI) using a 50:1 (protein:trypsin) ratio. RapiGest was cleaved and removed from the sample according to the manufacturer's protocol. Aliquots containing 1 mg of the digested sample were lyophilized and stored at -20 °C prior to use. The Sigma Aldrich 40 protein proteomics standard was prepared as instructed by the enclosed method. The total digest was desalted by C18 reversed phase SPE, subsequently evaporated to dryness and reconstituted in nano-pure water. Samples were corrected to 100 mM ammoniumacetate using 250 mM ammoniumacetate pH 4 or 5 shortly before injection.

2.3 Capillary Electrophoresis

A PA800 plus capillary electrophoresis (CE) system from Beckman Coulter (Brea, CA, USA) was used for all performed experiments. The PA800 plus is equipped with

a capillary cooling system using liquid cooling a power supply able to deliver up to 30 kV and a temperature controlled sample tray station. Neutrally coated capillaries were used for all experiments which were produced by coating the surface with a bi-layer of polyacrylamide. Capillaries were 95 cm (Figure 3-1) and 100 cm (Figure 3-3) in length and 150 µm outer diameter and 30 µm inner diameter were supplied by Beckman Coulter Inc. The used injection volumes and flowrates were calculated using a fluid viscosity of 1.04 cP and the Poiseuille equation. Finally, the BGE and LE consisted of 10% acetic acid and ammonium acetate (pH = 4 and 5 and 100 mM ionic strength), respectively.

2.4 Electrospray interfacing and Mass Spectrometry

The neutrally coated separation capillaries and mass spectrometry hyphenation were provided by Beckman Coulter Inc. (Brea, CA, USA) and produced by a previously reported method.[16, 17] A full description of both the sprayer and source can be found in the supplementary information. All experiments were performed on a MaXis 4G UHR-TOF or µTOF mass spectrometer from Bruker Daltonics (Bremen, Germany) at 1 Hz acquisition frequency. Dependent on the distance between the sprayer tip and the MS entrance, stable spray could be achieved between -900 and -1300 V ESI Voltage. Furthermore, the source temperature was set to 180 °C with the drying gas flow rate set to 2 L/min (nitrogen).

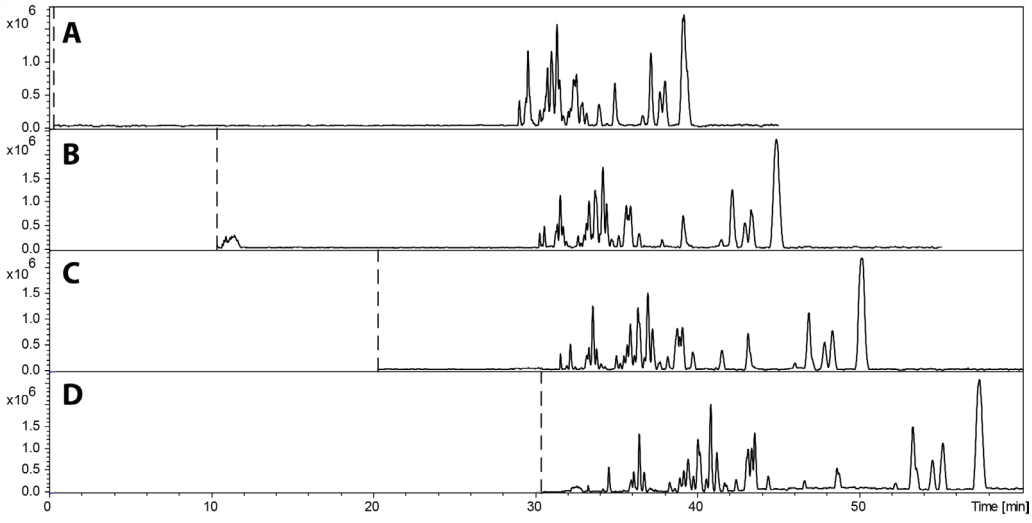

Figure 3-1: Neutral capillary (95 cm) t-ITP-CZE-MS analysis of 2,5 ng milk digest (37% capillary fill, 20 psi for 90 s) Base Peak electropherogram. Mass spectrometry; ESI voltage of -1050V; detection range 300-2900 m/z, other experimental conditions described in Materials and Methods (A) application of 0.8 psi at the inlet coinciding with the application of separation Voltage (B) 10 minutes of separation voltage before application of pressure (C) 20 minutes of separation voltage before application of pressure (D) 30 minutes of separation voltage before application of pressure.

3 Results and discussion

The application of t-ITP is an accepted way to improve CE loadability. However, the improved loadability comes at expense of the resolving power, as the injection of a large sample volume reduces the effective capillary length. Moreover, the high amount of leading electrolyte required for complete stacking of the analytes results in a wide plug of leading electrolyte migrating before the analytes further reducing the separation power. [18] To investigate whether a combination of t-ITP-CZE and Zero-Flow has a positive effect on the resolving power we have used bovine milk tryptic digest as a model sample. Figure 3-1A shows a typical t-ITP-CZE experiment with an injection volume of 37% of the separation capillary followed by separation at 25 kV and 0.8 psi directly applied at the start of the separation. At those conditions an effective separation window of only 10 minutes was recorded. Figure 3-1B-D represent experiments where 'Zero flow' periods (10, 20 and 30 minutes) were employed before the application of hydrodynamic pressure for mobilization and the flow rate required for ESI. With the application of increasingly longer periods of Zero Flow, an increase of the efficient separation window from 10 to almost 25 minutes can be observed. A schematic depiction of the observed effect is shown in Figure 3-2. Here we show the large volume injection of sample containing leading electrolyte into the capillary and the difference between the separation when pressure is applied directly with the current (D) or after a considerable wait (Zero-flow) period (E).

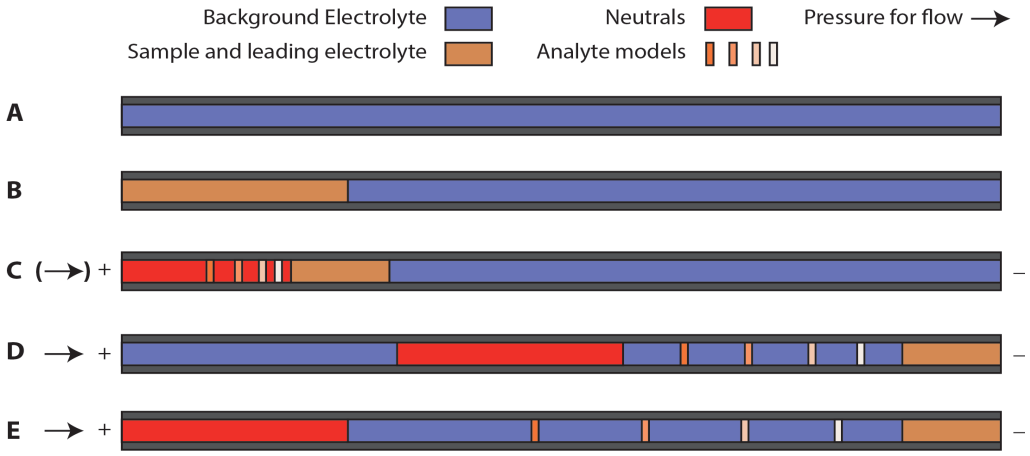

Figure 3-2: Abstract depiction of event inside the separation capillary during separation. (A) Capillary is filled with Back Ground Electrolyte (B) Large volume of sample mixed with leading electrolyte is injected. (C) Voltage is applied and pressure is applied here for normal operation. Pressure for flow is omitted for Zero-Flow operation. (D) Distribution of neutrals, analytes and leading electrolytes in normal operation. (E) Distribution of neutrals, analytes and leading electrolytes in Zero-Flow operation. At this time pressure is applied for induction of electrospray.

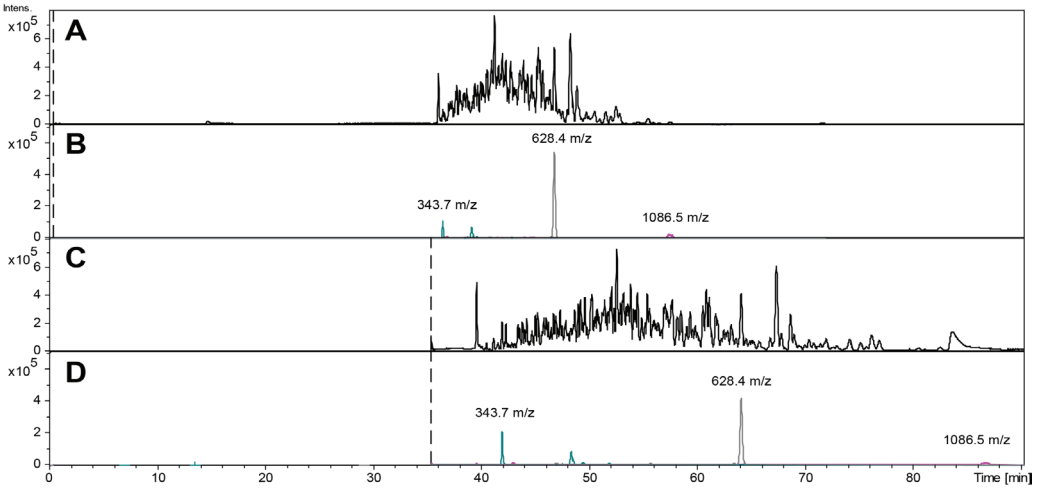

Figure 3-3: Neutral capillary (100 cm) t-ITP-CZE-MS analysis of 40 protein digest (34% capillary fill, 20 psi for 90 s) Base Peak electropherogram. Mass spectrometry; ESI voltage of -1100V; detection range 300-2900 m/z, other experimental conditions described in Materials and Methods (A) application of 1 psi at the inlet coinciding with the application of separation voltage (B) Extracted ion chromatrograms used for peak capacity calculations (C) 35 minutes of separation voltage before application of pressure (D) Extracted ion chromatrograms used for peak capacity calculations Zero-Flow separation.

Although the effect observed in the analysis of the milk digest is promising, the practical value of the Zero-Flow approach obviously needed to be assessed by the use of a significantly more complex sample. For this experiment a 40 protein tryptic digest was used, a 34% capillary volume plug was injected and 1 psi hydrodynamic pressure applied for mobilization and ESI spray, either immediately or after the zero-flow period. A comparison was made between a standard t-ITP-CZE experiment and the application of pressure after a Zero-Flow phase of 35 minutes. Figure 3-3 shows that the application of the Zero-Flow strategy leads to a significant increase in separation window when compared to the standard t-ITP-CZE procedure (47 vs. 22 minutes). The peak full widths at half maximum (FWHM) were measured for three empirically selected peaks of m/z 343.7; 628.4; 1086.5 respectively (beginning, middle and end of electropherogram; Figure 3-3B + D) and used to determine the average FWHM. Values of 0.233 and 0.250 minutes for the standard t-ITP-CZE and Zero-Flow respectively show that the increase of the efficient separation window is achieved without significant peak broadening. Taking the separation windows and the average FWHM into account, the application of the zero-flow approach increased the peak capacity from 94 to 188.

4 Conclusions

The performed research shows that the use of electrophoretic separation at stagnant separation medium conditions can be performed using a sheathless interface whereby greatly increasing the resolving power of capillary electrophoresis while using t-ITP for large volume injections. This is in contrast to previously used strategies for Zero-Flow CE-MS (sheathliquid and liquid junction) which inevitably result in loss of sensitivity due to dilution of the analytes before ESI.

The zero-flow strategy can of course also be used with normal CZE (1-2 % capillary volume injected). Here, however, the practical impact of the Zero Flow separation will be much lower, as the full capillary is already in use for separation and no leading electrolyte plug is present.

For further development, and obtaining even greater resolving power and sample loadability in CE-ESI-MS, capillaries with a greater capillary length will have to be tested. By using such capillaries of greater length a larger sample load should be possible, although the reduced potential over the capillary could result in increased diffusion and thereby loss in resolution. The use of larger diameter capillaries combined with the porous sheathless interface also shows some promise. [19] One significant drawback to the use of zero flow separation is the loss of all compounds with a pI below the BGE pH due to negative electrophoretic mobilities; therefore the use of a low pH BGE is advised.

Although, here this approach was only applied in a proof of principle manner to the separation of protein digests, in principle this approach is applicable to any type of sample suitable for CE separation as long as additional resolving power and loadability is required.

Acknowledgements

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Chapter 4

CE-MS for proteomics: advances in interface development and application (2007-2011)

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Abstract

Capillary electrophoresis-mass spectrometry (CE-MS) has emerged as a powerful technique for the analysis of proteins and peptides. Over the past few years, significant progress has been made in the development of novel and more effective interfaces for hyphenating CE to MS. This review provides an overview of these new interfacing techniques for coupling CE to MS, covering the scientific literature from January 2007 to December 2011. The potential of these new CE-MS interfacing techniques is demonstrated within the field of (clinical) proteomics, more specifically “bottom-up” proteomics, by showing examples of the analysis of various biological samples. The relevant papers on CE-MS for proteomics are comprehensively summarized in tables, including, e.g. information on sample type and pretreatment, interfacing and MS detection mode. Finally, general conclusions and future perspectives are provided.

1 Introduction

One of the main goals of proteomic studies is to achieve the highest possible proteome coverage. To this end, a multitude of multidimensional strategies have been evaluated during the last decade [1], with the main goal of introducing extra selectivity at various stages of the proteomic workflows so that the highest peak capacity and ultimately the most comprehensive analysis can be performed. In this context, miscellaneous separation/fractionation techniques, either chromatographic (ion exchange, size exclusion chromatography, reversed-phase liquid chromatography (RPLC) at different pH conditions, etc.) or electrophoretic (SDS-PAGE, iso-electric focusing (IEF), etc.) have been implemented as a first separation or fractionation step. However, reversed-phase liquid chromatography-mass spectrometry (RPLC-MS) is still the core technology for peptide and protein identification and it certainly will remain so in the foreseeable future [2-7]. For an update of the state-of-the-art of LC-MS for proteomics we refer to the review of Di Palma et al. in this issue of the Journal of Proteomics.

As the preferred final separation step in proteomic workflows RPLC offers numerous advantages, but as any analytical technology it has its limits. The most important ones are related to its separation mechanism – RPLC is a clearly suboptimal technique for small but also for rather large peptides, as these peptides are either not retained or irreversibly retained on conventional reversed-phase columns. Furthermore, at the flow rates at which RPLC is commonly operated, acidic and/or hydrophilic peptides are most vulnerable to ion suppression phenomena [8-10]. Finally, miniaturization of the LC-MS proteomics workflow is not an easy task and even the miniaturized workflows require at least 104 cells[11], which can be considered as a limitation when the analysis of really volume/material restricted samples is required [5].

In this review, we will concentrate on a different technology – namely, capillary electrophoresis–mass spectrometry (CE-MS). In CE, charged compounds in a fluid are separated from each other on the basis of their charge-to-size ratio under the influence of an electrical field. The first CE experiment in a fused-silica capillary with an internal diameter of 75 μm and a length of 100 cm was performed by Jorgenson and co-workers in 1981[12]. Since then, CE has emerged as a highly efficient separation technique and has, for example, played a central role in the deciphering of the Human Genome [13].

Nowadays, CE is considered as a powerful technique for the analysis of peptides as it exhibits a selectivity that is based on charge-to-size ratio differences and, therefore, in

comparison to RPLC, CE can provide complementary information on the composition of a biological sample. Furthermore, CE separations are highly efficient, relatively inexpensive (simple fused-silica capillaries vs. LC columns) and requires only small amounts of sample and reagents. As a result, the coupling of CE and MS is very attractive as it combines high-resolution separations with high detection selectivity and sensitivity. Still, the use of CE-MS in proteomics has lagged behind LC-MS. This might be due to the reduced migration time reproducibility which is occasionally observed as a result of sample-induced variations in electro-osmotic flow (EOF) often in combination with the use of non-appropriate washing conditions between runs and/or the poor concentration sensitivity compared to RPLC-MS.

However, since January 2007, a considerable number of papers have been published which report on the development and application of CE-MS for proteomics studies using electrospray ionization (ESI) and matrix assisted laser-desorption ionization (MALDI) ionization. In this review, we will summarize these studies and pay specific attention to emerging technological developments mainly related to the use of new interfaces for coupling CE to MS, enabling new possibilities for proteomics studies. We will focus on CE-MS-based bottom-up proteomics, i.e. the analysis of (complex) peptide mixtures obtained after enzymatic digestion of the protein sample. CE-MS for top-down proteomics, which concerns the analysis of intact proteins, is not covered in this review . Special attention is devoted to the advantages of CE-MS over RPLC-MS, e.g., operation at ultra-low flow-rates, capacity for miniaturization, and separation of polar and charged peptides, which often are poorly retained on RPLC columns. Selected illustrative examples are discussed in detail and some general conclusions and future perspectives are provided.

2 CE-MS methodology and applications

2.1 General aspects

CE has emerged as a highly efficient separation technique and, in effect, is used in a number of separation modes, such as capillary zone electrophoresis (CZE), normally referred to as “CE”, capillary electrochromatography (CEC), micellar electrokinetic chromatography (MEKC) and capillary iso-electric focusing (cIEF). In MEKC micelles are used as pseudo-stationary phases in the BGE allowing the simultaneous separation of neutral and charged compounds. However, the coupling of MEKC to MS is not straightforward and sensitivity of this combination remains limited. In cIEF, amphoteric

compounds, such as peptides, are separated according to their iso-electric point (pI) in a pH gradient formed by a mixture of carrier ampholytes (CAs) under the influence of an electric field[14]. After this so-called focusing step, the mobilization step is used to transfer the focused analytes toward the detector. Various strategies can be employed here but the most popular are the hydrodynamic and the chemical mobilizations, each providing various benefits. Major advantages of cIEF are related to its very high resolution capabilities and to the fact that this is a very efficient pre-concentration technique where the full separation capillary is initially filled with the sample to separate. In the scope of using it in conjunction with MS, the main limitation is the low volatility of the CAs (see section 2.2.2 for more details).

Detection in CE is most often based on on-column UV absorbance as it is easy to implement and it is widely applicable. However, it lacks both sensitivity, caused by the small optical path length as a result of the capillary diameter, and selectivity, as UV detection hardly provides any structural information of the analytes. For that reason, mass spectrometry has become increasingly popular as detection technique for CE. However, the coupling of CE to MS is not straightforward as a closed electrical circuit is required to maintain the high voltage across the capillary necessary for CE separation. Consequently, a CE–MS interface should provide a means to apply a voltage to the capillary outlet while insuring the independence of each of the electrical circuits (CE and ESI). The hyphenation of CE with MS started circa two decades ago, when Smith and co-workers introduced the first working interface[15]. Since then, research on this topic has been very active resulting in the development of various interfaces for several types of ionization sources[16-18].

For MS-based proteomics, ESI and MALDI are the two most widely used ionization methods. As different as they are, both ionization methods share one common trait, namely the ability to generate ions from large nonvolatile species without significant fragmentation. The hyphenation of CE and ESI-MS, however, appears to be more straightforward than CE and MALDI [19, 20]. With regard to CE-MS coupling, the composition of the BGE is the main constraint. The combination of CE and ESI-MS is compatible as both techniques are very well suited to compounds that can form ions in solution; yet if high buffer concentrations, nonvolatile constituents and/or surfactants are used as BGE, ion suppression effects are non-avoidable. Moreover, nonvolatile constituents may cause source contamination and high background signals. Therefore, only volatile BGEs like formic acid or acetic acid at low-pH (<3) conditions (most peptides migrate then toward the MS) using ESI in positive ionization mode are typically used for

CE--MS-based proteomics studies [21-24].

Method stability is of pivotal importance for a proteomics workflow. In CE adsorption of matrix components and/or analytes to the capillary wall may cause changes of the EOF which, consequently, leads to negative effects on the migration time reproducibility. Especially, when bare fused-silica (BFS) capillaries are used, separation efficiencies may be compromised as a result of adverse analyte–capillary wall interactions [25, 26]. Under these circumstances, it is important to implement systematic and rather extensive/ harsh washing conditions between consecutive runs to allow the analysis of biological samples with minimal sample pretreatment. Another way to address this problem is to modify the inner capillary walls of BFS capillaries with polymers, either neutral or positive ones. This approach has been successfully applied in various CE-MS-based proteomics studies [25-27]. Particular attention has to be given to the stability of the coating under the used experimental conditions as any leakage of the used polymer toward the MS can usually not be tolerated. For this reason, covalent/permanent, rather than non-covalent/ dynamic coating is preferred[28].

In the following sections, recent developments in CE-MS interfacing are discussed and exemplified for proteomics studies. As stated in the Introduction, only applications will be covered which concentrate on the use of CE-MS methodologies for bottom-up proteomics studies. This definition implies the analysis of (complex) peptide mixtures obtained after enzymatic digestion of the protein sample. Therefore, this section will not include the analysis of intact proteins which has been comprehensively reviewed by Haselberg et al. [29].

2.2 Sheath-liquid CE-MS

2.2.1 Coaxial interfacing

CE can be coupled to MS via a sheath-liquid or a sheathless interface, but for a variety of reasons the sheath-liquid interface has been so far the most widely used for CE–ESI-MS in proteomics studies [30-32]. Sheath-liquid interfaces use a coaxial sheath-liquid that mixes with the CE effluent. The aim of the sheath-liquid is to provide electrical contact between the CE separation and the ESI source. The sheath-liquid interface was first developed by the group of Smith[15]. In this configuration, the separation capillary is inserted in a tube of larger diameter in a coaxial setting (Figure 4-4). The conductive sheath-liquid, to which the CE terminating voltage is applied, is administered via this

outer tube and merges with the CE effluent at the capillary outlet. In most configurations, a gas flow is applied via a third coaxial capillary in order to facilitate the desolvation process. The sheath-liquid can be used to optimize the ESI process and, therefore, the composition and flow rate of the sheath liquid are critical parameters in optimizing the overall performance of the sheath-liquid CE-MS method. The sheath-liquid is often composed of a mixture of water and organic modifier, such as methanol or isopropanol, containing a volatile acid, such as formic or acetic acid. The operating flow rates in CE are usually between 20 and 100 nL/min, while the sheath-liquid flow rate typically ranges from 1 to 10 µL/min. Thus, the flow rates of the sheath-liquid are at least one order of magnitude higher than the intrinsic CE flow rates which results in compromised ionization efficiencies and sensitivities of the analytes [24, 33, 34]. Additionally, the sheath-liquid can, to some extent, influence the peak efficiency. It has been shown that while the EOF presents a flat profile, the addition of both sheath-liquid and sheath-gas introduces a parabolic flow component which decreases the achievable peak efficiency [25, 35, 36]. The application of a counter pressure at the inlet of the separation capillary has been proposed to reduce this effect [25, 35, 36]. In general, the sheath-liquid interface can be considered as a fairly robust platform. This and the fact that sprayers are commercially available together might explain why the coaxial sheath-liquid design is so commonly used in CE-MS-based proteomics [37, 38]. An overview of CE-MS-based proteomics studies using a sheath-liquid interface over the period from January 2007 to December 2011 is given in Table 4-1. Selected examples are discussed below.

Catai et al. demonstrated that the use of a sheath-liquid interface in CE-MS for peptide analysis may cause some extra band broadening when compared to the results obtained with CE-UV [25]. The observed band broadening for the peptides was caused by both the hydrodynamic capillary flow induced by the nebulizer gas and the data acquisition rate of the ion trap mass spectrometer used. High separation efficiencies for peptide analysis could be obtained by cancellation of the hydrodynamic flow caused by the nebulizer gas and by using a sufficiently high-data acquisition rate.

CE coupled to TOF-MS via a sheath-liquid interface was used for the analysis of O- and N-glycopeptides of a tryptic digest of recombinant erythropoietin by Gimenez et al.[39]. The adsorption of glycopeptides to the inner surface of a bare fused-silica capillary wall was prevented by rinsing the capillary with 1 M acetic acid between runs. By using this washing procedure, RSDs (%) of migration times improved from ~3% to ~0.5%. It was demonstrated that the presence of reference compounds in the sheath-liquid had a detrimental effect on the detection of the peptides and glycopeptides of the protein digest

and as such the authors suggested that depending on the complexity of the sample to be analyzed, the use of references in the sheath-liquid can be inconvenient for the detection of the analytes of interest by CE-TOF-MS.

Elhamili et al. evaluated monoquaternarized piperazine, 1-(4-iodobutyl) 4-aza-1-azoniabicyclo[2,2,2] octane iodide (M7C4I) as a surface derivatization reagent for CE in combination with TOF-MS for the analysis of protein digests [26]. The M7C4I piperazine, at alkaline pH, forms a covalent bond via alkylation of the ionized silanols producing a cationic surface with a highly stable and reversed EOF. The obtained surface yielded fast separations (<5 min) of peptides at acidic pH with high separation efficiencies (up to 1.1 x 106 plates/m) while no bleeding of the coating reagent was observed into the MS instrument. The potential of CE-TOF-MS using M7C4I-coated capillaries was demonstrated for the analysis of protein digests.

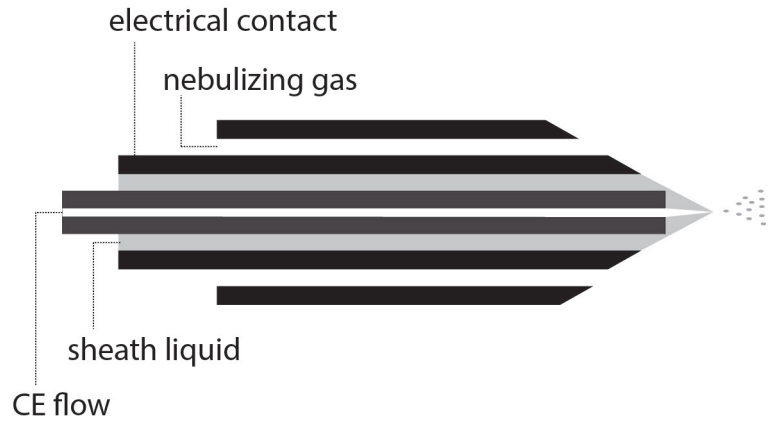

Figure 4-4: Schematic depiction of the coaxial sheath-liquid design as published by Smith and coworkers[15]

Tempels et al. developed an on-line SPE-CE-MS system for the analysis of peptides. Analytes were preconcentrated on a C18 microcolumn (5 x 0.5 mm id), which were then introduced into the CE system via a valve interface [40]. The CE system with a Polybrene-poly(vinylsulfonate) bilayer coated capillary was combined with an ion-trap MS via ESI using a co-axial sheath-liquid sprayer. The on-line coupling of the SPE and CE step by the valve interface allowed an independent functioning of the system parts. LODs for selected peptides were in the range of 1.5-3 ng/mL. The potential of the on-line SPE-CE-MS system was demonstrated by the analysis of a cytochrome C digest. However, some hydrophilic peptides did not show sufficient retention on the SPE column, and were lost during pre-concentration.

2.2.2 Liquid-junction interfacing

Another approach to couple CE to MS is via a liquid-junction interface, which was first reported by Henion and co-workers [41]. In a liquid-junction interface, the CE capillary and ESI emitter are separated by a small gap in which the CE effluent mixes with a sheath-liquid before entering the ESI emitter [34, 41, 42]. The mixing of the sheath-liquid with the CE effluent serves two purposes. The first is modifying the mixture of the BGE to make it more compatible with ESI-MS analysis. Secondly, and most importantly, it is used to create the closed electrical circuit required for CE separation. There are different solutions for the placement of the electrode required for a closed CE circuit and application of the ESI voltage. For example, the group of Dovichi used coated separation capillary ends [42] and sheath-liquid application [43-45] from a grounded vial (Figure 5-1 on page 96), while the group of Chen used a stainless steel ESI emitter as both ESI and CE electrode. The flow-rates of the sheath-liquid used in liquid-junction designs are typically in the range of 60 to 200 nl/min, which is significantly lower than the flow-rate used in coaxial sheath-liquid interfaces. The group of Dovichi recently described an interface based on electrokinetic pumping of the sheath-liquid and capable to operate in the nano-electrospray regime [44]. As a result, the system provides great flexibility in separation buffers, and allows the use of uncoated and inexpensive capillaries for separation. Low attomole range (high picomolar concentration range) LODs for selected peptides were reported.

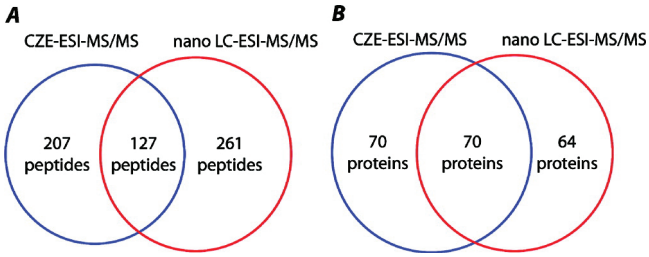

Figure 4-5: Venn diagram illustrating the overlap of peptides and proteins identified by both CZE-ESI-MS/MS and UPLC-ESI-MS/MS at 95% confidence (FDR < 0.15%): (A) peptides, (B) proteins. Taken from [45].

Recently, Li et al. evaluated the potential of CE coupled via an electrokinetically pumped sheath-liquid interface to ESI-MS for the analysis of a tryptic digest of a sample of intermediate protein complexity, the secreted protein fraction of Mycobacterium marinum [45]. For CE analysis, 11 fractions were generated from the sample using RPLC; each fraction was analyzed by CE-ESI-MS/MS, and 334 peptides corresponding to 140 proteins were identified in 165 min. In comparison, 388 peptides corresponding to 134

proteins were identified in 180 min by triplicate UPLC-ESI-MS/MS analyses, each using 250 ng of the unfractionated peptide mixture. Overall, 62% of the peptides identified with CE-ESI-MS/MS and 67% of those identified with UPLC-ESI-MS/MS were unique (Figure 4-5). CE-ESI-MS/MS favored basic and hydrophilic peptides with low molecular masses. Combining the two data sets increased the number of unique peptides by 53%.

Until now, only a few proteomic studies have been carried out with CE-MS using liquid-junction interfacing. However, the results obtained so far are promising and certainly will stimulate further development.

2.3 Sheathless interfacing

Sheathless interfacing is, in principle, the most straightforward way to couple CE to ESI-MS as the fundamental property of CE, i.e. the intrinsically low flow-rate, is used in its most optimal way. Therefore, various sheathless interfaces have been developed to enhance the concentration sensitivity of CE-MS [24, 46-51]. In the sheathless interface configuration, the CE voltage is directly applied to the CE buffer at the capillary outlet. This can be achieved by applying a metal coating to the end of a tapered separation capillary or by connecting a metal-coated, full metal or conductive polymeric sprayer tip to the CE outlet. Another way to make a closed circuit is by insertion of a metal micro-electrode through the capillary wall into the CE buffer end or by direct introduction of a micro-electrode into the end of a CE capillary [47]. While most of these interfaces have been proven to provide very high performances, mainly in terms of sensitivity, the main bottleneck has been the manufacturing complexity or the long-term stability of the sprayers. Concerning the latter parameter, the main limitation of, for example, metal coated tips is related to the stability of the metal coating which, upon operation, may degrade significantly till a point where the electrical contact is lost. A clogging is another frequent issue of these approaches, especially if a tapered tip used.

Moini developed a sheathless interface design consisting of a CE capillary with a porous tip as nanospray emitter[24]. The porous tip, being the outlet of the separation capillary, approach is very straightforward and does not require any fluidic connection. In this configuration, the properties of the glass portion of the capillary which has been etched with hydrofluoric acid provide electrical contact between the lumen of the CE capillary and the outside without the need for any liquid flow through the pores of the porous tip. To operate this interface, part of the porous portion of the tip is inserted in a buffer reservoir on which the electrical connections are being made. Besides the relative simplicity of the

approach, the major attributes of this strategy are the use of non-tapered tips (30 µm ID) and the displacement of the electrolysis reactions outside from the separation capillary, which are significant advantages, as compared to previous sheathless interfaces. This interface was used for the identification of proteins in complex mixtures and the separation of protein–protein and protein–metal complexes of erythrocytes, showing its potential for proteomic studies [24, 52, 53].

Based on the sheathless interfacing concept of Moini, a prototype high-sensitivity porous sprayer (HSPS) sheathless interface for CE–ESI-MS was recently developed by Beckman Coulter (Figure 5-2 on page 99). Busnel et al. used this novel approach for the analysis of tryptic digests of bovine serum albumin (BSA) and E. coli.[54]. It was found that the interface is capable of generating a stable spray with flow-rates ranging from below 10 nL/min to >340 nL/min, enabling its use in either the mass or concentration-sensitive

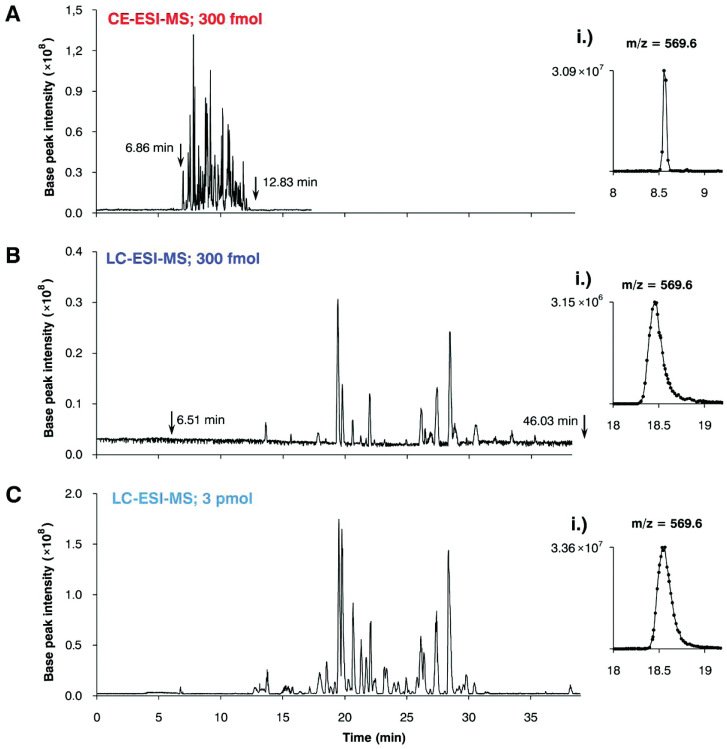

Figure 4-6: Base peak electropherogram and base peak chromatograms of rat testis H1 histones digested with endoproteinase Arg-C using (A) CE–ESI-MS, sample amount 6.15 ng (300 fmol); (B) LC–ESI-MS, 6.15 ng (300 fmol); (C) LC–ESI-MS, 61.5 ng (3.0 pmol). CE conditions were as described in Figure 5. LC–ESI-MS was performed using a homemade fritless column: packed 10 cm with 3 µm reversed-phase C18 (Reprosil). The gradient (solvent A, 0.1% formic acid; solvent B, 0.1% formic acid in 85% acetonitrile) started at 4% B. The concentration of solvent B was increased linearly from 4% to 50% during 50 min and from 50% to 100% during 5 min. A flow rate of 250 nL/min was applied. (i) Extracted mass trace of the ion m/z = 569.6 corresponding to the triply charged peptide KALAAAGYDVEKNNSR. Each dot represents a single full scan. Taken from [56].

region of the ESI process. The incorporation of transient isotachophoresis (t-ITP) in this approach increased the mass loading of the system resulting in concentration limits of detection in the sub-nanomolar range for selected peptides. The possibility to operate this interface at very low flow rates allowed the use of non-charged neutral capillary coatings at a flow rate below 10 nl/min, which resulted in a peak capacity above 320 for the separation of an E. coli tryptic digest. In addition to providing a very high sensitivity, the operation of this interface at such low flow rates reduces ion suppression effects normally observed when separation techniques (LC or CE) are hyphenated to ESI-MS using higher flow rates(> 200 nL/min)[55].

The group of Lindner has recently also evaluated the suitability of this sheathless CE-MS approach for peptide analysis [56]. Three different cationic capillary coatings were investigated for stability, resolution, and EOF and were found to enable reproducible separations by CE–ESI-MS. The sheathless CE–ESI-MS method was compared with nano-RPLC–ESI-MS by analyzing Arg-C-digested rat testis linker histones. With comparable amounts of sample injected, the number of identified peptides increased by more than 60% with sheathless CE–ESI-MS (Figure 4-6). Lindner et al. demonstrated that low-molecular mass peptides (<1400 Da) were preferentially identified by CE–ESI-MS, as this group of peptides poorly interacts with the reversed-phase material in the nano-LC system. This selectivity difference remained valid even with 10 times more sample loaded in the LC system than in the CE system.

The nanoliter to microliter per minute flow rate used in microchip CE shows a strong potential for interfacing such devices with nanoflow ESI-MS sources. Microfluidic chips can be coupled directly to an ESI interface using pressure-driven or EOF to direct the liquid into the spray [57, 58]. Although the use of microfluidic devices has been under investigation for some time now, their use in proteomic studies has been very limited so far. The potential of microchip CE-ESI-MS for proteomics has recently been investigated by Mellors et al. [59]. They developed a microchip CE system with an incorporated ESI sprayer, which required no junctions and therefore created no additional dead volume. As the developed spray tip required a significant EOF, a positively charged covalent capillary coating was used to create a reversed and strong EOF under which a stable spray could be achieved for the analysis of a BSA digest. With this system, plate numbers of 200,000 could be generated with peak widths around a second at half height. Overall, the developed approach shows a strong potential for proteomic studies as high peak capacities could be achieved in a narrow separation window.

2.4 Hyphenation of CE and MALDI-MS

In a nutshell the difference between the CE-ESI-MS and CE-MALDI-MS comes to a simple fact: the ionization process in MALDI is physically decoupled from the separation process. As a result, the influence of the BGE composition on the ionization is lower in MALDI and, if a correct ionization matrix is chosen, a higher ionic strength BGE can be used [60-62]. Since MALDI-MS generally operates under high vacuum conditions, offline approaches have so far been preferred. Although these approaches introduce a certain discontinuity to the workflow, they also permit to envisage the integration of on-plate reactions, which can be as diverse as enzymatic digestion, chemical modification or enrichment of subclasses of compounds.

While commercial CE instruments enable fraction collection, in practice this often leads to breakdown of the CE current occurring at each movement of the outlet reservoir, which induces a loss of peak efficiency. As a result, the potential applicability of these approaches is very limited. Several research groups have been developing home-made interfaces to couple CE and MALDI-MS, with one of the prerequisites being that these interfaces do not provoke any current interruption so that the high efficiency capabilities of CE can be preserved for the MALDI-MS detection. Interfaces based on the use of a T-junction, allowing the addition of a flow of sheath-liquid at the outlet of the separation capillary have for example been proposed [62]. T-junction based interfaces are suitable to most CE conditions with or without EOF; however, it has been demonstrated that, as in ESI-MS, the creation of a parabolic flow profile in the separation capillary using the addition of a sheath-liquid at the outlet of the separation capillary can compromise the peak efficiencies. As of today, T-junction interfaces are certainly the most common ones as they can be combined in a rather straightforward manner to commercial MALDI spotters. An alternative to T-junction based interfaces is the liquid-junction interface, however, a significant challenge with this strategy is to optimize the configuration to minimize dead volumes, which can, when they are too large, dramatically deteriorate the resolution. In an effort to provide a simple and powerful coupling of CE with MALDI-MS, an iontophoretic fraction collection approach has recently been proposed [60]. It is based on electromigration and molecular diffusion and does not rely on the use of any sheath-liquid, liquid-junction nor superimposed hydrodynamic pressure. The main advantage of this approach is that it is compatible with both bare fused-silica and neutrally coated capillaries and that the in-capillary resolution is totally independent from the spotting process. As a result, as many fractions as required can be collected without deteriorating the achievable resolution.

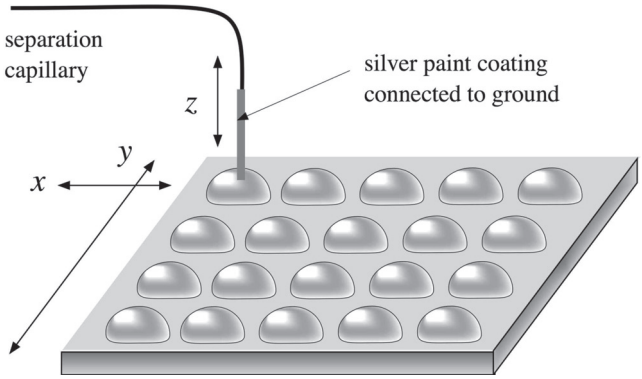

Figure 4-7: Schematic of the robotized fraction collection interface including a MALDI plate mounted on an x,y-stage and a separation capillary silver-coated at the tip for a connection to ground and mounted on a z-axis holder. Taken from [60].

In all the approaches described above, the fraction collection process is discontinuous in the sense that analytes separated by CE are collected in discrete droplets. As a result any discrete spotting strategy will induce a certain loss of resolution as compared to the in-capillary resolution. In this context, some groups have been proposing continuous offline deposition approaches. For example, Reijtar et al. have proposed an approach where the CE effluent was collected on a stainless steel MALDI plate through offline vacuum deposition [63]. In this configuration, the CE separated analytes were deposited on 100 micrometers wide streaks and the electrical contact at the interface was provided by the use of a liquid-junction. More recently, Wang et al. proposed a similar continuous deposition approach [64], but here the electrical contact was not made through the use of large liquid-junction but through the use of an open fracture [65] at the outlet of the separation capillary. Although the fabrication of the open fracture is not straightforward, the continuous aspect of the deposition presents a lot of capabilities, especially in view of the significant hardware and software advances which have recently been made in the field of MALDI imaging.

A number of CE-MALDI-MS applications have been developed and used for proteomics over the past few years, which are summarized in Table 4-4. Although most of them are mainly of academic nature, a few relevant cases will be discussed in detail to illustrate the potential of CE-MALDI-MS for proteomics. Zuberovic et al. performed an analysis of human CSF using a PolyE-323 positively charged coated capillary coupled to a sheath-liquid MALDI spotter [66]. The described strategy was also applied for quantitative proteomics in CSF after incorporating an iTRAQ labeling step in the sample preparation [67]. Absolute quantification could not be performed, but with the used strategy 43 proteins in CSF could be identified which varied in concentration after a traumatic brain injury.

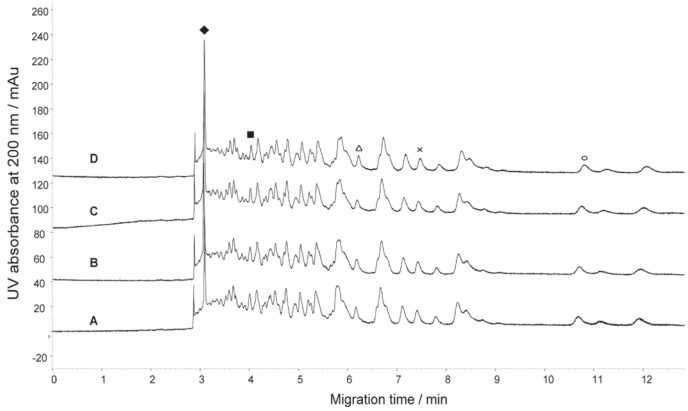

Figure 4-8: Impact of spotting interval time on separation resolution. Experimental conditions: HPC-coated capillary, total/effective length 80/21.5 cm × 50 µm i.d.; voltage, 30 kV; current, 12 µA; UV absorbance at 200 nm; BGE, 10% acetic acid; sample, tryptic digest of α-lactalbumin (35 µM) and β-lactoglobulin (27 µM); sample injection, 30 mbar 30 s. (A) No spotting. (B) Spotting every 60 s. (C) Spotting every 30 s. (D) Spotting every 15 s. Taken from [60].

Busnel et al. developed a novel CE-MALDI spotting interface, shown in Figure 4-7, to achieve efficient fractionation at very low flow-rates using separation in a neutrally coated capillary [60]. The use of a coated capillary tip instead of a liquid-junction of sheath-liquid interface to achieve a closed circuit allowed fractionation onto a pre-structured MALDI plate enabling concentration into a small matrix spot (Figure 4-8). This application was tested on an 8 protein digest and compared to direct MALDI analysis of the same sample mixture, resulting in 87 peptides detected after MALDI fractionation compared to only 36 with direct analysis. Although an increase of almost 150% in the number of detected peptides was found the average sequence coverage only increased from 24% to 38%. A downside of the developed strategy was the need for sampling/spotting into larger volume droplets of MALDI matrix resulting in analyte dilution.

2.5 Multidimensional systems

The use of a single separation technique for the analysis of a complex sample such as protein digests of whole cell lysates or tissue lysates is often not sufficient. The larger is the number of peptides obtained after enzymatic digestion of a complex biological sample, the higher is the risk of co-migration if a single dimension separation approach is used. This, in turn, may lead to the difficulties in the interpretation of the MS/MS spectra of co-migrating peptides. The use of two complementary techniques generally results in an improved peak capacity thereby reducing co-migration of compounds. In this section, relevant applications of on-line and off-line multidimensional separation approaches are discussed. An overview is given in Table 4-2.

Chen et al. [68] coupled cIEF on-line to nano-RPLC-ESI-MS for the analysis of tryptic digests of Formalin-fixed and paraffin embedded (FFPE) tissue and yeast membrane [69-71]. The aim was to achieve maximum peptide coverage by coupling two complementary separation methods (Figure 4-9). A total of 14,478 distinct peptides were identified from micro-dissected FFPE glioblastoma tissue, leading to the identification of 2733 non-redundant SwissProt protein entries. For yeast tryptic digest, a total of 33,772 peptides and 3433 proteins could be determined after using a number of different sample preparation strategies. Although it was claimed that the sample consumption for this method was low, sample consumption for such a 2D technique will obviously be higher than for a single dimension separation technique.

A similar strategy was used by Fang et al. for the analysis of human saliva and mouse brain mitochondrial proteome [72, 73]. Their strategy consisted of an off-line coupling of an ITP/CZE to nano-RPLC-ESI-MS via a fraction collector. Like cIEF, ITP/CZE is enabling r in-capillary sample concentration for the use of larger volume injections, but it provides better separation of the peptide bands reducing the appearance of peptides in multiple CE fractions. In a comparison with the previously described cIEF-LC method it was found that the developed ITP/CZE-LC-MS method was superior in the detection of both

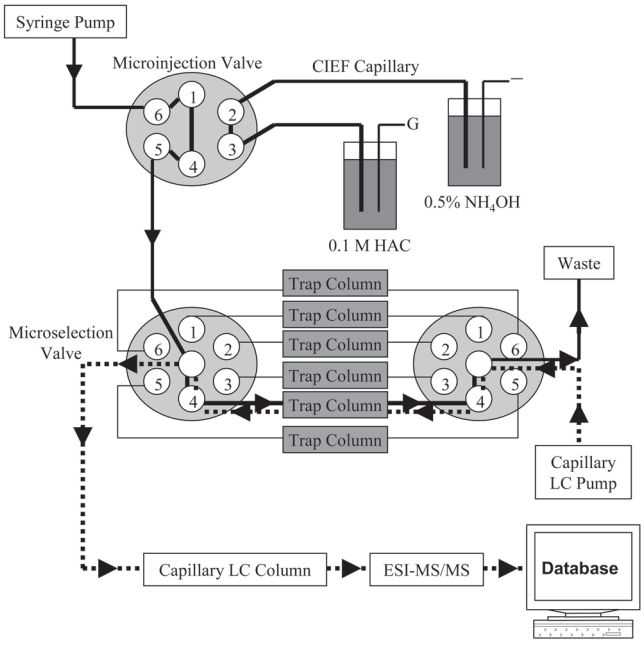

Figure 4-9: Schematic of on-line integration of CIEF with CRPLC as a concentrating and multidimensional separation platform. Solid and dashed lines represent the flow paths for the loading of CIEF fractions and the injection of fractions into a CRPLC column, respectively. Taken from [68]

peptides and proteins. Moreover, the use of both methods resulted in complementary data and resulted in an even higher number of detected mouse mitochondrial peptides and proteins.

An off-line CIEF-LC-MS method for the analysis of ovarian endometrioid adenocarcinoma was developed by Dai et al. [74] The developed strategy was used to compare the signaling pathways in two closely related carcinoma cell lines. A total of 2612 proteins could be identified of which only 1092 could be determined in both cell lines. For one of the cell lines 1749 proteins could be identified after merging the data from three analyses, but only 311 of these proteins could be identified in all three runs while 561 could be identified in only two runs leaving almost half of the proteins identified in just one run.

In principle, cIEF and CZE are not orthogonal separation techniques and, therefore, the coupling of the two methods will not yield much additional information for whole digests. Nevertheless, when separation of intact proteins is performed by cIEF followed by a digestion step and subsequent analysis by CZE the increase in information could

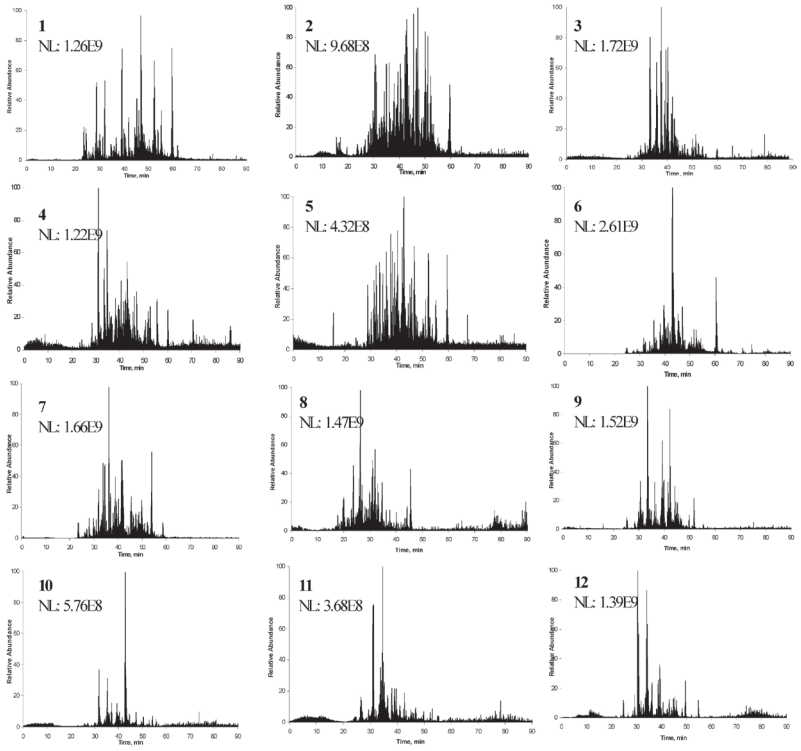

Figure 4-10: Base peak chromatograms of a representative CIEF/CRPLC multidimensional separation of 9.6 µg of yeast tryptic peptides obtained from the soluble fraction of cell lysates. Each number represents the sequence of CIEF fractions further analyzed by CRPLC from acid to basic pHs. Taken from [68].

be significant. Using this strategy the most abundant proteins can (at least partially) be isolated from the less abundant compounds. This coupling of CIEF-CZE was used by Hanrieder et al. for the proteomic analysis of human follicular fluid [75]. In their method the intact protein content was first fractionated by cIEF into 10 fractions ranging from pH 3 to 10, which were then digested, and separated by CZE using a positively charged coated capillary, and this was finally followed by spotting on a MALDI target plate for subsequent analysis.

A method using iso-electric trapping (IET) in membrane separated wells and histidine as buffering electrolyte was developed by Cologna et al. [76] The investigation showed that histidine can serve as a very suitable buffer over a wide pH range and that the coupling of IET and CZE can have some benefits. So far, the method was only applied to BSA digested before the IET phase of the separation. As previously discussed, IET and CZE are not orthogonal methods and their coupling in this fashion will most likely not improve the sequence coverage when compared to an efficient single dimension separation strategy. If it could be proven that the IET strategy could also be used for the separation of whole proteins, then the coupling of these methods in a more orthogonal strategy comparable to the method developed by Hanrieder et al. [75] should be possible.

2.6 Relevant work in adjacent fields

Although the analysis of native peptides is a highly specialized area and is in many ways different from mainstream proteomics work it is difficult to ignore this important fraction of the literature. The group of Mischak et al. have used a CE-ESI-MS method that has been extensively characterized [23] and applied to the study of human urine [77-80], CSF [81] and bile [82]. The aim of their studies was to identify native peptide biomarkers for a large variety of diseases. In their strategy, CZE separation is coupled to TOF-MS through a co-axial sheath-liquid interface. Their CE-TOF-MS approach has been used for the analysis of thousands of biological samples, demonstrating the robustness of their approach for large-scale clinical studies. A comprehensive review of the use of CE-MS in peptidomics/proteomics for the discovery of biomarkers has recently been published by Mischak et al. [83].

3 Conclusions and outlook

Over the last 5 years, the hyphenation of CE with MS has significantly benefitted from technological developments in the field: today the coupling of CE to a mass spectrometer may be considered as simple and straightforward as coupling of a LC instrument to MS. The operation at ultra-low flow rates, capacity for miniaturization, and the ability to separate efficiently (highly) polar and charged peptides are the three main distinctive features of CE-MS. Although the loadability has always been a topic of discussion with regard to the general applicability of CE, novel in-capillary concentration methods and the gain of sensitivity due to the ultra-low flow-effects have reduced the relevance of this issue.

The next question is whether proteomics researchers are ready to use CE-MS for their applications and whether modern proteomics offers research questions that will be typically well-suited for a CE-MS approach. In that respect, we foresee that an exploration of the above mentioned ultra-low flow rate effects and the analysis of volume restricted samples will be a main area of CE-MS applications in proteomics. As discussed in this review, ultra-low flow conditions not only provide sensitivity but also has the advantage that under these circumstances ESI behaves “as a detector with almost equimolar responses for samples prepared from a complex serum matrix” [55]. Miniaturization is another strong aspect of CE-MS. Scaling down the LC-MS routines is an expensive and challenging process, CE, on the other hand, is a “nano-technique” by its nature.

Although CE-MS has until now been used primarily with relatively non-complex samples, we hope that this review will contribute to a more general appreciation of the possibilities of this technique and challenge the proteomics community to implement it in their workflows, and we hope to see the results of these experiments in the near future. Although single cell analysis might be considered the Holy Grail in proteomics, even hundreds of cells, which will still not be amenable to more conventional proteomic workflows, in principle provide enough material for a comprehensive CE-MS based proteomic analyses.

Sample	BGE/ampholytes	Coating	MS interface	Mass analyzer	Remarks	Ref.
Cytochrome C, β-lactoglobulin A and ribonuclease B tryptic digests	1 M Formic acid adjusted to pH 2.0 with NH4OH	-	Sheathliquid ESI 3 µL/min 50/50 (v/v) MeOH/H2O	IT	large volume injection with pH junction	[84]
Cytochrome c tryptic digest	20/80 ACN/20 mM Ammoniumformate (v/v)	Open Tubular n-octadecyl- and cholesterolbonded surfaces	Sheath-liquid ESI 50/50 (v/v) 1 mM Ammonium Acetate/MeOH	IT		[85]
Cytochrome C tryptic digest	200 mM Formic acid adjusted to pH 2.5 with NH4OH	Polybrene and poly(vinyl sulfonate)	Sheath-liquid ESI 75/25 (v/v/v) ACN/water with 0.1% Formic Acid	IT	On-line SPE using a column switching system	[40]
BSA tryptic digest	10 mM Ammonium Acetate pH 3.0	monoquaternarized piperazine (positive coating)	Sheath-liquid ESI 2 µL/min 80/20 (v/v) MeOH/H2O with 10mM Acetic Acid	TOF		[26]
Erythropoietin and biosimilars tryptic digest	20 mM Ammonium Acetate pH 7.0	-	Sheath-liquid ESI 3.3 µL/min 60/40 (v/v) iPrOH/H2O with 0.5% Formic Acid		In-line immuno affinity SPE	[86]
BSA tryptic digest	30 mM Ammonium Acetate pH 3.0	N-Methylpolyvinylpyridium (positive coating)	Sheath-liquid ESI 2 µL/min 80/20 (v/v) MeOH/H2O with 10mM Acetic Acid	TOF		[87]
Soy bean extract tryptic digest	0.5 M Formic Acid	-	Sheath-liquid ESI 3 µL/min 50/50 (v/v) iPrOH/H2O	TOF		[88]
Recombinant human erythropoietin (rhEPO) tryptic digest	50 mM Formic Acid and 50 mM Acetic Acid	-	Sheath-liquid ESI 3.3 µL/min 50/50 (v/v) iPrOH/H2O	IT		[39]
Four bovine protein tryptic digest	10 mM ammonium acetate pH 7.0	-	Electrokinetically junction at the tip ESI 50/50 MeOH/ H2O 10 mM acetic acid	Orbitrap		[44]

Table 4-1: Overview of CZE-ESI-MS proteomics applications reported between 2007 and 2011.

Sample	First dimension	Second dimension	Remarks	Ref.
Human Saliva	Transient-Isotachophoresis using neutral hydroxypropyl cellulose coating. 2% Sigma Aldrich Pharmalyte as leading electrolyte and 100 mM Acetic acid as BGE	Nano-RPLC-ESI-MS linear gradient acetonitrile/water	tITP/CZE-LC coupling performed off-line using CE fraction collector equipped with sheath-liquid tip to obtain a closed circuit.	[73]
Formalin-fixed and paraffinembedded (FFPE) tissues	cIEF using neutral hydroxypropyl cellulose coating and 2% Sigma Aldrich 3-10 ampholyte	Nano-RPLC-ESI-MS linear gradient acetonitrile/water 0.02% formic acid	cIEF-LC coupling performed by on-line column switching system. Protein digestion performed before cIEF	[69]
Yeast membrane tryptic digest	cIEF using neutral hydroxypropyl cellulose coating and 2% Sigma Aldrich 3-10 ampholyte	Nano-RPLC-ESI-MS linear gradient acetonitrile/water 0.02% formic acid	cIEF-LC coupling performed by on-line column switching system. Protein digestion performed before cIEF	[70]
Yeast membrane tryptic digest	cIEF using neutral hydroxypropyl cellulose coating and 2% Sigma Aldrich 3-10 ampholyte	Nano-RPLC-ESI-MS linear gradient acetonitrile/water 0.02% formic acid	cIEF-LC coupling performed by on-line column switching system. Protein digestion performed before cIEF	[71]
Mouse brain mitochondrea	Transient-Isotachophoresis using using neutral hydroxypropyl cellulose coating. 2% Sigma Aldrich Pharmalyte as leading electrolyte and 100 mM Acetic acid as BGE	Nano-RPLC-ESI-MS linear gradient acetonitrile/water	tITP/CZE-LC coupling performed off-line using CE fraction collector equipped with sheath-liquid tip to obtain a closed circuit	[72]
Ovarian endometrioid adenocarcinoma cell tryptic digest	cIEF using neutral hydroxypropyl cellulose coating and 2% Sigma Aldrich 3-10 ampholyte	Nano-RPLC-ESI-MS multi phase linear gradients acetonitrile/water 0.02% formic acid	cIEF-LC coupling performed off-line using fraction collector equipped with T-piece make-up flow and stainless steal needle to obtain a closed circuit. Protein digestion performed before cIEF	[74]
Human follicular fluid	cIEF in 1% RioRad 3-11 ampholyte	CZE-MS in unmodified capillary and 10 mM acetic acid BGE. Fraction collection followed by MALDI-TOF MS/ MS	cIEF-CZE coupling performed off-line using hydrodynamic fraction collection. Protein digestion performed after cIEF fractionation	[75]
BSA tryptic digest	Iso Electric Trapping (EIT) using Poly(vinyl-alcohol) based buffering membranes	CZE-MS with neutral polyacrylamide coating and 250 mM Formic Acid BGE. Stacking performed with histidine as leading electrolyte. Detection by MALDI-TOF-MS	EIT-CZE coupling performed off-line. Protein digestion performed before EIT. MALDI spotting performed using sheath-liquid interface to provide closed circuit and MALDI matrix	[76]
Mycobacterium marinum secreted protein tryptic digest	RPLC using water:acetonitrile gradient followed by fractionation	CZE-ESI-MS using unmodified capillary and sheath-liquid interface	RPLC-CZE coupling performed off-line. Protein digestion performed before RPLC fractionation. MS analysis performed with Orbitrap	[45]

Table 4-2: Overview of multidimensional proteomics applications reported between 2007 and 2011.

Sample	BGE/ampholytes	Coating	MS interface	Mass analyzer	Remarks	Ref.
Six protein tryptic digest	0.1% Polybrene in 0.1% acetic acid	-	Sheathless porous tip interface	IT		[24]
BSA and *E.coli* tryptic digest	10 % acetic acid pH 2.2	polyacrylamide (neutral coating)	Sheathless porous tip interface	TOF	Large volume injection with tITP	[54]
Histone H1 endoproteinase Arg-C digest	0.1% Formic Acid pH 2.7	M7C4I, PolyE-323 and PEI (Positive coatings)	Sheathless porous tip interface	Orbitrap		[56]
BSA tryptic digest	50/50 (v/v) MeOH/0.4% Acetic Acid	PolyE-323 (positive coating)	Sheathless on-chip ESI sprayer	QTOF		[59]

Table 4-3: Overview of CZE-ESI-MS proteomics applications reported between 2007 and 2011.

Sample	BGE/ampholytes	Coating	MS interface	MALDI Matrix	Remarks	Ref.
E-coli tryptic digest	250 mM formic acid with 5 mM ammonium phosphate pH 2.2	-	MALDI spotter using make-up flow to provide matrix and electric circuit	CHCA	Large volume injection through stacking	[89]
BSA Myoglobin tryptic digest	10 mM acetate pH 4.25	1,2-dilauroyl-sn-phosphotidylcholine (neutral coating)	MALDI spotter with hydrodynamic spotting and no make-up flow	CHCA	Large volume injection using pH junction	[90]
Cerebrospinal fluid tryptic digest	10 mM Acetic Acid	PolyE-323 (positive coating)	MALDI spotter using make-up flow to provide matrix and electric circuit	HCCA		[66]
8 Protein tryptic digest	10% Acetic Acid pH 2.2	Hydroxypropylcellulose (neutral coating)	MALDI spotter with conductive coating on spotter tip for continuous separation voltage	CHCA	Large volume injection using ITP	[60]
a-lactalbumin tryptic digest	10 mM Ammonium Bicarbonate pH 9.0	-	MALDI spotter using make-up flow to provide matrix and electric circuit	CHCA		[91]
Cerebrospinal fluid tryptic digest	10 mM Acetic Acid	PolyE-323 (positive coating)	MALDI spotter using make-up flow to provide matrix and electric circuit	HCCA	Quantification with iTraQ labeling	[67]
6 protein tryptic digest	40mM Triethylamine to pH 2.3 with phosphoric acid and 20% v/v ACN	Fullerenol and polylatex diol (neutral coating)	MALDI spotter using make-up flow to provide matrix and electric circuit	CHCA		[92]
3 protein tryptic digest	80mM phosphoric acid, 40mm TEA (pH 2.3) and 20% v/v ACN	Polylatex diol (neutral coating)	MALDI with hydrodynamic spotting and voltage assisted spotting and no make-up flow	DHB	Large volume injection using ITP	[93]

Table 4-4: Overview of CZE-MALDI-MS proteomics applications reported between 2007 and 2011

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Chapter 5

CE-ESI-MS for bottom-up proteomics: advances in separation, interfacing and applications

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Mass spectrometry Reviews 2014

Abstract

With the development of more sensitive hyphenation strategies for Capillary electrophoresis-electrospray- mass spectrometry the technique has reemerged as technique with high separation power combined with high sensitivity in the analysis of peptides and protein digests. This review will discuss the newly developed hyphenation strategies for CE-ESI-MS and their application in bottom-up proteomics as well as the applications in the same time span, 2008 to present, using co-axial sheathliquid.

Subsequently all separate aspect in the development of a CE-ESI-MS method for bottom up proteomics shall be discussed, highlighting the discusses applications and discussing pros and cons of the various choices. The separation of peptides in a capillary electrophoresis system is discussed including the great potential for modeling of this migration of peptides due to the simple electrophoretic separation process. Furthermore, the technical aspects of method development are discussed, namely; background electrolyte choice, coating of the separation capillary and chosen loading method.

Finally, conclusions and an outlook on future developments in the field of bottom-up proteomics by CE-ESI-MS will be provided.

1 Introduction

The potential of Capillary Electrophoresis (CE) as high efficiency separation technique was evident from the very first experiments performed by Jorgenson and co-workers in 1981 [1]. Since then, the “track record” of CE has grown enormously but its contribution to the deciphering of the Human Genome remains the most notable one [2]. CE was also the first liquid based separation technique to be coupled in-line to mass spectrometry [3-5]; however, it has never gained the wide spread acceptance in the field of bottom-up proteomics that liquid chromatography has enjoyed [6]. One of the reasons for the under representation of CE in the field of proteomics has been the lack of a sensitive hyphenation strategy that would allow for highly efficient separation under ultra-low flow rates. The complexity of samples that are obtained in bottom-up proteomics strategies are such that the speed of the current generation of mass spectrometers still requires wide separation windows with high peak capacity to obtain the desirable proteome coverage. In the case of CE, high separation power can only be achieved at minimal flow rates in the separation capillary and hyphenating CE and MS at such low flow rates was only possible using a sheath liquid interface [5]. An unfortunate side-effect of this coaxial sheathliquid interface is the strong dilution of the analytes before entering the mass spectrometer. As ESI-MS is generally considered a concentration sensitive technique this strategy results in strong decrease in potential signal intensity and thereby losses in sensitivity. Another well-known and often discussed weakness of CE-MS is the lack of loadability resulting in low sample concentration sensitivity.

Some significant developments have been made in the field of CE-MS proteomics allowing for improved loadability, separation power and sensitive electrospray ionization. In the field of hyphenation, a number of new interface designs have been developed which has resulted in a boost in interest for CE-MS as a viable analytical technique for bottom-up proteomics [7-10]. Although two methods, namely the porous sheathless interfacing [8] and the electrokinetic junction [11], were used in a significant portion of new papers in CE-MS proteomics field, a few additional designs will be discussed shortly.

The most regularly used CE separation mode, capillary zone electrophoresis (CZE), only separates analytes on their charge in solution and their spatial dimension, therefore the separation happens in a very straightforward manner. Moreover, peptides are built up out of the 20 standard amino acids resulting in a high capability for predicting electrophoretic mobility of a peptide with a known primary structure. The influence of post-translational modifications (PTMs) on a peptide are less straightforward to predict.

The addition of phosphorylation, glycosylation or oxidation can result in any number of charge and spatial size changes, but CE has proven to be able to efficiently separate the various modifications from their native form or even show the position of the modification [12-14].

This review will cover the recent applications of CE-ESI-MS in the field of CE-MS bottom-up proteomics (analysis of proteolytic digests); covering new hyphenation techniques as well as application of different separation buffers, capillary coatings and injection modes. The discussed applications will roughly cover the period of 2009 to 2014 although when necessary earlier reports are included. The emphasis will be on the compatibility of all aspects of the CE separation with ESI-MS hyphenation.

2 Interfacing

In general terms there are two methods for coupling CE with mass spectrometry. Intuitively, the sheathless interface where the separation buffer is the only liquid involved in the separation and ionization of all analytes appears to be the most straightforward method. The second and so far most widespread approach is the use of a make-up flow or sheathliquid which allows for the contact closure at the capillary outlet and also aids in ionization. Both approaches have been used in a large variety of different ways and each have their advantages and drawbacks which will be discussed in Section 2.5. Here we concentrate on the interfaces that were used for bottom-up proteomics purposes only, although some examples of interfaces that show great promise are discussed. When interested in the history of CE-ESI-MS the review by Maxwell et al. will cover all CE-ESI-MS interface designs [15].

2.1 Coaxial sheathliquid interfacing

The coaxial sheath-liquid interface was developed by the group of Smith and co-workers (Figure 4-4 on page 70) [5]. The setup requires the outlet of the separation capillary to be inserted into a conductive tube with the capillary end equal or slightly protruding the end of the conductive tube. The tube is made of stainless steel or platinum and through this the make-up flow/sheath liquid is delivered to the separation capillary outlet to generate a closed circuit and can be used to apply the spray voltage or ground (MS manufacturer dependent). A nebulizer gas flow is then applied through a second coaxial tube to generate a spray plume and improve de-solvation. The use of a sheath liquid gives the user stronger flexibility in the choice of BGE and the possibility for adding

an organic solvent to the sprayed solution (generally methanol or propanol) and a volatile acid (formic or acetic acid) which further aids in the ionization process. The sheath liquid flow rate is typically operated between 1 to 10 µL/min and the choice of the proper mixture is important to prevent moving boundaries in the separation which will deteriorate the peak shape and resolution. There are many conditions that can and need to be optimized in coaxial sheath liquid interfacing; separation capillary protrusion from the sheath liquid tube, sheath liquid consistency and flow rate, and finally, electrospray voltage.

As in-capillary flow rates are generally in the 20 to 100 nL/min range the use of a sheath liquid in the microliter range will inevitably cause dilution resulting in loss of signal intensity [16]. Moreover, the sheath-liquid can, to some extent, influence the shape efficiency [17]. Besides moving boundaries due to poorly chosen sheath liquid constituents, it was found that the addition of both sheath-liquid and sheath-gas introduces a parabolic flow component which decreases the achievable peak capacity. The application of a counter pressure at the inlet of the separation capillary has been proposed to reduce this effect [16-18]. Despite the drawbacks and complex optimization, the coaxial interface provides a very robust interface and for this reason it has been used most for bottom-up proteomics approaches [19-21]. A summary of coaxial sheathliquid CE-ESI-MS applications in bottom-up proteomics is given in Table 5-1.

Despite the strong history of the coaxial interface the number of publications in the field of bottom-up proteomics has decreased in recent years due to the development of a number of new interfaces which will be discussed in later sections. The coaxial interface is still frequently used in the discovery and analysis of native peptide biomarkers from body fluids; human urine [22, 23], CSF [24] and bile [25]. A comprehensive review on biomarker discovery addresses this topic [26].

2.2 Electrokinetic junction-at-the-tip

The electrokinetic junction was first introduced by the group of Norman Dovichi in 2010 as a nanospray sheath-flow interface [11] which in itself was an improvement of their previous nano-sheathliquid design [7]. The technique is named an electrokinetic junction as no added pressure is used to supply the sheathliquid and the only make-up flow that is added is the electroosmotic flow in the spray tip as a result of the spray voltage. This allows for a closed contact at low electrospray flow rates with minimal sample dilution. The actual make-up flow rates have not been measured making the dilution factor

somewhat unclear, and it is possible that this flow rate is influenced by the spray tip internal diameter. The relatively straightforward design of the sprayer (Figure 5-1) allows for the coupling of CE to any mass spectrometer with a nano-source.

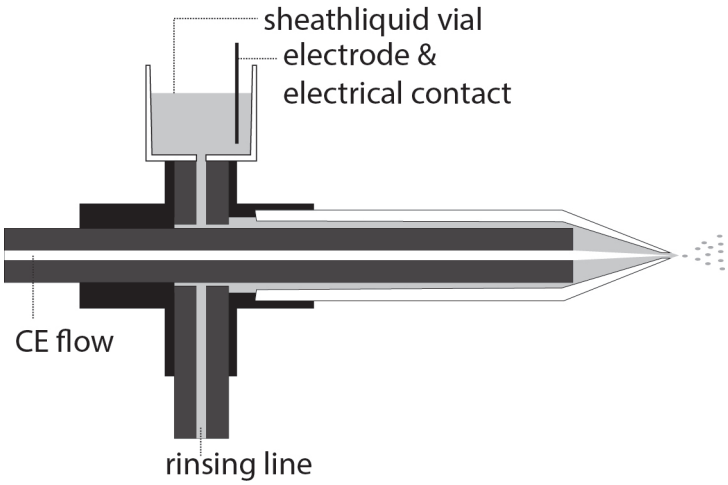

Figure 5-1: Schematic depiction of the electrokinetic junction nano sheathliquid interface as published by Dovichi and coworkers. [11]

The interface has been used for straightforward bottom-up proteomics of both mouse tumor cells [27] and Escherichia coli [28, 29] and Mycobacterium marinum secretome [30]. In two instances a reversed phase fractionation was employed, thereby reducing the sample complexity to obtain optimal proteome coverage. The fractionation of Mycobacterium marinum secretome was performed by RPLC followed by drying and reconstitution of the digest fractions resulting in the identification of only 334 peptides and 140 proteins [30]. The analysis of seven reversed phase SPE fractions of E.Coli resulted in the identification of 4902 peptide and 871 protein groups which shows the potential of the interface for in-depth proteome studies and demonstrates that the low number of proteins found in the Mycobacterium marinum secretome was a result of low sample complexity.

The electrokinetic interface was applied in the development of targeted [31] and quantitative proteomics strategies [32, 33]. In these cases the use of a triple quadrupole or quadrupole-iontrap type mass spectrometer was preferred over the proteomics field gold standard Orbitrap type. While the interface resulted in excellent sensitivity for the detection of specific peptides from standards or spiked into complex samples, the run to run signal reproducibility was relatively poor. Li et al report relative standard deviations

(RSDs) for repeated analysis of >20% which would not be sufficient for large scale analyses and is far from the RSDs that can be achieved with the coaxial sheath liquid interface.

The design of the interface and the simplicity of assembly makes it possible to do an off-line iso-electric focusing (IEF) separation before placing the outlet end of the capillary in the interface followed by mobilization and ESI-MS analysis [34-36]. While in the initial application a well-known ampholyte, PharmalyteTM, was used [34], in later applications it was found that a mixture of only six amino acids gave sufficient focusing for analysis [35, 36]. Both regular bottom-up proteomics of protein mixtures and mouse tumor cell lysate digests, and relative and absolute quantitative studies of rat cells using eight-plex isobaric tags were performed using this approach [35, 36].

The interface has also been used in conjunction with in-line and on-line enrichment and digestion techniques which could potentially improve proteome coverage and make CE-MS for proteomics more flexible in its application [37, 38] which will be discussed in the injection modes section.

Finally the interface was used in a diagonal separation strategy for the analysis of phosphopeptides from a complex sample [39, 40]. The setup uses two separation capillaries coupled by a Immobilized Alkaline Phosphatase micro-reactor which results in the removal of the phosphate groups from a phosphopeptide. Through this manner peptides with multiple phosphorylations could be found and identified without the negative influence of a neutral phosphate loss in CID fragmentation. One drawback is the loss of positional information of the phosphate group in the peptide which could be elucidated by the proper MS3 experiments.

It’s worthy of attention that only the separation capillaries with a maximum outer diameter of 150 µm have been used in all reported experiments with the electrokinetic junction. Although in comparison to traditional capillary diameters (> 300 µm) it is more difficult to produce a satisfactory end, a narrower capillary can be inserted farther into the pulled glass capillary that is used as the microvial and spray needle. By having the outlet end of the separation capillary closer to the spray tip the created micro-vial size is reduced to a minimum and thus reducing potential diffusion. On the whole, the use of the electrokinetic junction-at-the-tip interface shows great promise for more large scale applications. The relative simplicity of the interface makes it easy to use and evidently flexible in the application of a wide range of bottom-up proteomics applications.

2.3 Porous sheathless interface

Sheathless interfacing is in principle the most straightforward method to couple CE separations to a mass spectrometer through ESI [15]. Until recently, relatively high flow rates at the outlet contact represented a common weakness for all reported realizations of this coupling. When reducing the flow rate by lowering the pH in unmodified capillaries or by applying a neutral coating (section 5) bubble formation at the outlet electrode or contact, causes instable electrospray or even crashing current. To circumvent the problem of bubble formation in the separation capillary Mehdi Moini developed a porous sheathless interface, which allows for a closed contact through a part of the separation capillary that is porous to ions [8] (Figure 5-2). In this manner the electrode contact and therefore bubble formation happens outside the separation capillary and no current instability or crashing is observed. Therefore, separations at ultra-low flow rates (<10 nl/min) can be achieved resulting in high peak capacities and high sensitivity electrospray [41, 42].

The porous sheathless interface has been used by a number of groups for the analysis for proteolytic digests. It was shown that under the right conditions (coatings see, section 5) analysis can be performed at flow rates as low as 5 nl/min resulting in a peak capacity over 300 in a separation window of about 60 minutes. Through this, both optimal separation power and sensitivity can be achieved [41]. The group of Lindner compared a porous sheathless CE-ESI-MS method with nano-RPLC–ESI-MS by analyzing Arg-C-digested rat testis linker histones [43]. In these experiments it was found that using the sheathless CE-ESI-MS strategy more peptides with a mass below 1400 Da can be identified when compared to nano-RPLC–ESI-MS. Moreover, when an equal amount of material is loaded, CE-ESI-MS gives 60% more identified peptides than the nano-RPLC–ESI-MS method. The Yates group used the porous sheathless set up for the analysis of Pyrococcus furiosus tryptic digest using an extended separation capillary (from 90 to 190 cm) and an in-line solid phase micro extraction (SPME) column with incremented elution steps to significantly improve the number of identified peptides by 87 % over direct injection of the same sample amount [44]. Although not all peptides found by direct analysis could be found using the SPME method, this is most likely due to poor retention of these peptides on the reversed phase SPME column.

Where the Yates group simply extended the 30 micrometer internal diameter capillary [44] the Tang group coupled a porous sheathless sprayer to a capillary with a significantly bigger internal diameter (100 µm i.d.) [45]. The goal of using a larger bore capillary is to

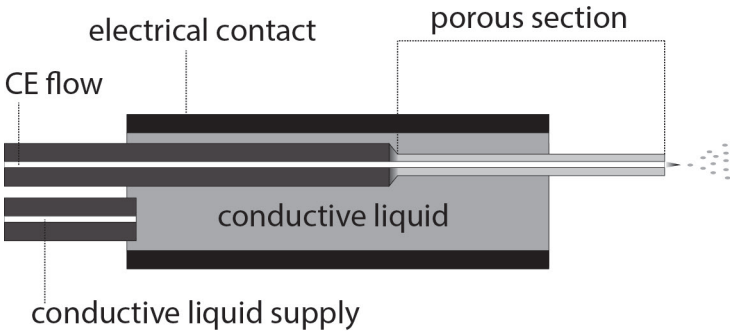

Figure 5-2: Schematic depiction of the porous sheathless interface as published by Mehdi Moini.[8]

improve loadability, and combined with transient isotachophoresis (t-ITP see section 6) the sample load could be increased to 2.5 microliters using a 33% capillary fill as sample load. The obtained method showed good linearity in a dilutions series but the observed RSDs of up to 22% are something that will need to be addressed.

The system was used in its most straightforward setup to characterize the monoclonal antibody TrastuzumabTM [46]. An unmodified capillary was used combined with a 1% capillary fill for sample loading to obtain full sequence coverage of both the light and heavy chain of the antibody. The post-translational modifications on the antibody (mainly glycosylation) were also investigated through MS2 to determine the different glycoforms present in the Trastuzumab formula.

Separation and ionization at very low flow rates were also investigated and showed significant improvement in ionization efficiency of phosphorylated peptides [42]. This improvement in ionization efficiency was further explored by the Lindner group in an investigation of post translational modifications of human histones [47]. It was found that under optimal conditions a factor 100 less material is needed in the porous sheathless CE-ESI-MS protocol than was needed in nano-LC to obtain similar numbers of modified peptides. The sheathless CE-ESI-MS method identified a total of 52 modified peptides divided over phosphorylation, acetylation and deamidation and combinations of the three.

Moini and Martinez developed a ultrafast CE separation system on basis of the porous sheathless interface using short and narrow bore (5 µm) separation capillaries[48]. The setup was built up out of a 96 well plate positioned vertically into an in house fabricated holder for easy access of the separation capillary to the sample and separation buffers. Electrokinetic injection was used to inject samples and full analysis only required a

maximum of 5 minutes. Although in early development stages, an evolution of this setup could provide very high throughput analysis in the future.

Generally, the porous sheathless interface for CE-ESI-MS results in significant improvements in sensitivity over more traditional (coaxial sprayer) methods. The potential of electrospray at very low flow rates results in extreme separation power combined with very high sensitivity. There is a lack of exploration of the run to run reproducibility of the signal intensity for peptide analysis for this setup, although it was shown in a study of antigen specific IgG glycopeptides that peak area ratio RSDs below 20 % could be achieved [13].

2.4 New developments

The vast majority of recent publications in the field of CE-ESI-MS bottom up-proteomics have been based on the electrokinetic junction or porous sheathless interfaces. Two noteworthy developments have been made in the field of CE-ESI-MS hyphenation recently by the groups of Chen and Her respectively.

The Chen group published a nano sheathliquid interface which they have dubbed the Junction-at-the-tip micro flow through vial [9, 49, 50]. The interface consists of a stainless steel hollow needle with very specific dimensions (Figure 5-3) including a beveled tip at a 60 degree angle from the plane in which the outlet end of the separation capillary is placed. The beveling produces a very sharp edge which creates a very high local electric field resulting in good ESI conditions. The principle of the needle is very similar to that of the electrokinetic-junction-at-the-tip interface with the difference that the sheath liquid is supplied hydrodynamically from a grounded vial (it can for example be the outlet buffer vial in the CE system). The interface was applied in the analysis of standard protein, peptide and amino acid mixtures. In the analysis of amino acids, improvements in limits of detection between a factor 2 and 13 were reported using CZE separations [9, 51]. It was separately applied to the analysis of released glycans from fish serum protein [52]. As with the Electrokinetic junction-at-the-tip this interface can be used to facilitate IEF separations. In the application by Zhong et al. small proteins were separated in an in-line fashion, meaning that the capillary did not have to be removed from the interface between IEF and mass spectrometry analysis [50]. As the interface is relatively new and is being used by only two groups, few papers have been published and it has not been used for bottom-up proteomics work as of yet.

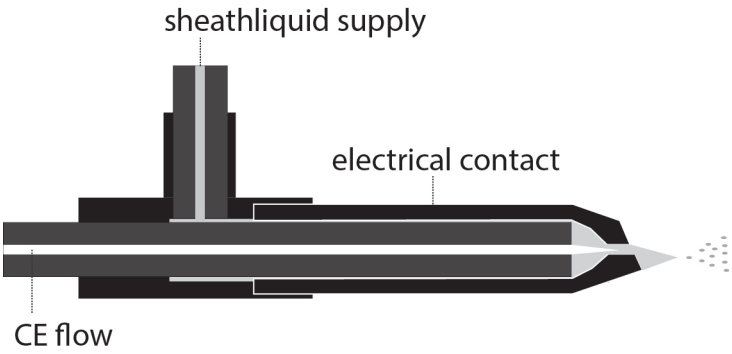

Figure 5-3: Schematic depiction of the junction at the tip interface as published by Chen and coworkers.[9]

Wang et al. present an interface that is very similar to that reported by the Dovichi group, here called the electrokinetic junction-at-the-tip interface. It consists a pulled fused silica spraying tip with the separation capillary threaded through the spray needle with the exit as close to the spray orifice as possible. In this setup however the sheathliquid is not supplied electrokinetically but through pressure. The spray orifice is also etched using hydrofluoric acid to increase the i.d. which reduces the potential for clogging between runs. The setup was applied in a proof of principle study which determined that the setup gave a linear response with regard to concentration and was capable of doing t-ITP as a means of injecting larger sample volumes. [33]

Wang and Her published the development of a sheathless CE-MS interface using a robust poly(dimethylsiloxane) membrane emitter with conduction through means of a liquid-film. The interface employed a 125 µm-thick triangular emitter tip with a 50 µm inner diameter channel. There is a conductive liquid reservoir through which the contact is made. The separation capillary is inserted into a 375 µm channel the connection to the emitter runs through the conducting reservior. Stable electrospray could be achieved from 30 to 350 nL/min emitter flow rates. The interface was applied to the analyses of a a five-peptide mixture in low-EOF (60 nL/min) and high-EOF (210 nL/min) conditions and showed the stability and versatility of the emitter [10]. The same group of Wang and Her adapted their interface so a counterflow can be applied to the outlet side of the capillary and thereby accommodating counterflow electrokinetic supercharging (see sample loading Section 6)[53]. This interface design has only been used and reported by this group and no applications in bottom-up proteomics have been reported to date.

2.5 Interfacing general aspects

At the moment none of the existing techniques for the coupling of CE and (ESI) MS is dominating the field of CE-MS bottom-up proteomics. The low flow rates that can be achieved in porous sheathless interfacing resulting in high sensitivity could be seen as superior feature[41, 42, 54, 55]. Busnel et al. also showed that by decreasing the flowrate in the separation capillary strongly improved peak capacities can be achieved. [41] This effect is now feasible using the porous sheathless interface. Nevertheless, achieving similar peak capacities using any of the sheathliquid interfaces is also possible. For the nano sheathliquid interfaces it is even possible to achieve separation under zero-flow conditions achieved by neutral coating of the capillary wall [28, 56].

However, the use of a sheathliquid in any form affords the user significantly more flexibility with regards to the analysis conditions. The inability to use IEF separation (Section 6.3) and restrictions in the types of buffer that can be used in the CE separation when using sheathless interfacing (Section 4) are factors to consider when choosing between a sheathless or sheathliquid approach. As sheath-liquid can change the pH conditions at the outlet such that part of the dilution effects can be negated and can be even further reduced by the addition of an organic solvent. As no direct comparison of the signal intensities and/or identified number of proteins and peptides from the same sample has been performed between the various sheathless and sheathliquid interfaces it is unclear what the real influence of the sheathliquid is on the obtained results.

3 Peptide separation by CZE

As the most common CE separation mode used for proteomics purposes is Capillary Zone Electrophoresis (CZE) we will focus on the behavior of peptides under these conditions. Generally the 20 amino acids are divided up in five groups being; basic, acidic, non-polar, polar and hydrophobic. In the case of CE separations, the individual influences in the electrophoretic mobility of the non-polar, polar and hydrophobic peptides are very similar. Naturally, depending on the pH of the BGE (see section 4) the basic and/or acidic residues dominate the influence on electrophoretic mobility over the other amino acids. As a result of only a few amino acids having significant and comparable influence on migration time shift, a density plot of a CZE separation of a mixture of peptides will show a distinct lined pattern. Figure 5-4 shows the CZE separation at pH

2.2 of a complex tryptic digest with clearly discernible bands that can be identified as resulting from the number of basic amino acids (lysine, arginine or histidine) that were present in the identified peptide. A separate example of this effect for native peptides separation was found in search for biomarkers from body fluids using CE-MS [57].

As a result of the small influences of amino acid size and shape on the electrophoretic mobility of a peptide a number of models have been developed which only employ known values on the individual amino acids for the prediction of electrophoretic mobility. In the simplest semi-empirical models only the C-terminal acid and N-terminal amine and side group pKa and BGE pH are used to determine the charge of the peptide. There are two different approaches for the determination of peptide size. The first approach is considering amino acids as links in a classical polymer in solution thereby disregarding the size and shape of the functional moieties [58]. An alternative approach is using the molecular weight of the peptide as an indication of the total size, which does take side group size into account to certain extent [59]. Rickard et al. also used alternative pKa's than those known for individual amino acids as the peptide bond influences both the C terminal carboxylic acid and N-terminal amine [59]. Hilser et al. showed that these adapted peptide pKa values result in significant improvement of mobility prediction over the free solution amino acid pKa's [60]. Although a number of these models have been developed there is no consensus on the 'best' one. A comparison of models by Tessier et al. showed that depending on the sample type (mainly short or longer peptides) different models can provide a better fit to your data [61]. The semi-empirical models are relatively easy to implement and have over the years proven useful in a number of studies in the verification or identification of peptide structures [62-64].

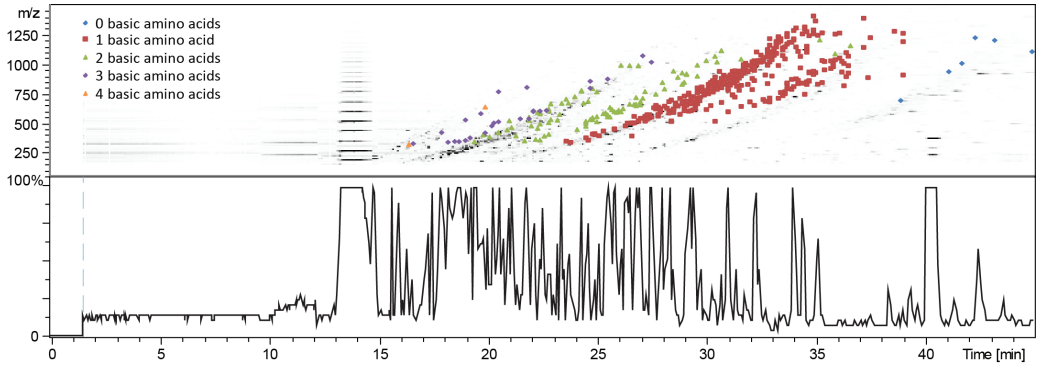

Figure 5-4: Separation of a complex cell lysate digest at pH 2.2. (A) shows the density plot of the observed masses in MS1 with the peptides that are identified and their number of basic amino acids. (B) Corresponding base peak electropherogram.

A second approach for the development of migration time models is the use of experimental knowledge on the individual amino acids and potentially even their position in an amino acid chain [65-67]. These 'blind' models are built up using a least squares calculation, thereby building up knowledge of the influence of the individual amino acids on the migration/elution time of a peptide. Thus far, this approach has only been used to predict peptide RPLC retention times but is highly applicable to CZE-ESI-MS peptide separation data. A drawback to this approach is the requirement of a very complex sample to build a suitable model. Amino acid position (terminal or middle) and the number of basic and/or acidic amino acids could be considered as additional factors in this approach as Grossman et al. have shown that electrophoretic mobility does not increase linearly with charge [58]. The only requirements for the development of such a model are computational power and a sufficiently rich data set. When separation conditions remain consistent model of this type should be applicable to any type of peptide, making it unnecessary to build a new model when a different protease is used for digestion.

Analysis of PTMs, of which phosphorylation and glycosylation are the most important, is another area of the proteomics where the number of CZE applications is steadily growing. While CZE is regularly applied in the analysis of released glycans [68] and whole glycosylated proteins [69], the analysis of glycosylated peptides by CE-MS is relatively unexplored with only a few publications in recent years. CZE can very efficiently separate the varying glycoforms as the sugars are rather bulky and significantly alter the analytes Stokes radius and thereby influencing the electrophoretic mobility. The incorporation of one or more sialic acids, which has a pKa of 2.6, into the glycan structure will significantly change peptide electrophoretic mobility. Figure 5-5 shows the separation of IgG1 derived glycopeptides and the influences of the different sugar moieties on the separation can be observed including a significant shift in migration time for glycopeptides with a sialic acid.

Phosphorylation has an effect on electrophoretic mobility of a peptide similar to the addition of a sialic acid. A phosphorylation has two pKa's at ±1.2 and ±6.5 (amino acid

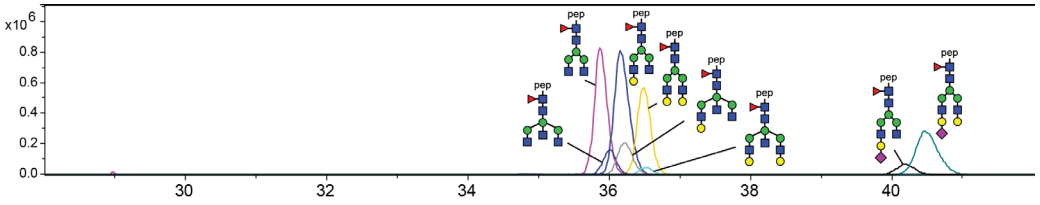

Figure 5-5: Separation of glycopeptides derived through IgG1 tryptic digest. (Figure adapted from [13])

dependent) which result in either one or two extra negative charges on a phosphorylated peptide depending on the choice of BGE pH. An enzymatic and biologically meaningful phosphorylation usually takes place on serine, threonine or tyrosine; thus a peptide can contain more than just one potential phosphorylation site. Unfortunately, the identification of the specific modification site by just mass spectrometry data is not very straightforward. Recently, Dong et al. showed that using CZE, it is possible to separate phosphorylated peptides in a site specific manner [14]. In their investigation Dong et al. used 4 peptides with the same amino acid sequence but varying phosphorylation states to show the effect of phosphorylation on migration time. As expected the peptide without a phosphorylation, with one phosphorylation and two phosphorylations separated at low pH. It was also found that the peptides with different positional phosphorylations could be almost completely resolved. Although this is a specific example of one amino acid sequence it is possible that this effect could be consistent and could be applied in phosphorylation site identification when mass spectrometry data is found to be insufficient. It is also possible that this effect is very much dependent on the peptide length and it's amino acid sequence; therefore this effect might not be universal for all variations of phosphorylation sites on all peptides.

4 Separation buffers

As can be seen from the application summaries (Table 5-1, Table 5-2 and Table 5-3) generally only volatile buffer components are used in separation buffers. Although the use of non-volatile components might work in the short term, it will inevitably lead to contamination of the source resulting in inconsistent results or low sensitivity. The buffers of choice usually consist of either formate or acetate as anion and ammonia as cation for CZE separation. In a few cases carbonate is used as anion although the risk of bubble creation through the production of CO2 is then present [31, 38, 70].

The choice of a separation buffer for CE-ESI-MS depends not only on the target analytes but also on the type of the interface. A sheathliquid or junction-at-the-tip interface (section 3) offers significantly more flexibility with regard to the chosen buffer components and especially separation pH (Table 5-1 and Table 5-2). The range in chosen buffer pH using these interfaces runs from < pH 1.0 [14] to up to pH 8.0 [31, 38]. Although low pH in the spray solution if preferred for positive ion-mode ionization of peptides this low pH can be achieved by the added sheathliquid. It is however important to pay attention the compatibility of the BGE and the sheathliquid to prevent moving boundaries in the separation medium which could result in reduction of separation efficiency. The BGE

pH mainly influences electrophoretic mobilities of peptides with aspartic and glutamic acids in their sequence as these have a pKa of about 4.5 [58 , 71]. At low pH (<3.5) the influence of the acidic residues on peptide electrophoretic mobility is minimized and migration will be mostly driven by the number of basic residues (Figure 5-4).

When a sheathless interface is used for CE-ESI-MS hyphenation there are some significant restrictions on the applicable BGE components. As the separation buffer is also the only liquid that is being utilized in the ESI process, the low pH is needed to ensure satisfactory ionization. As a result, formic and acetic acid are most commonly used as sole buffer constituent as the addition of cationic buffer components (ammonia) can result in clustering and signal suppression during the ionization process. The use of very low pH buffers has an added advantage to the separation power when using unmodified fused silica capillaries. The low pH results in almost full protonation of the free silanols, thereby strongly reducing the EOF and optimizing separation power.

The addition of an organic solvent to the BGE has been reported in a small number of recent applications [14, 33, 44]. It is however not common practice in either sheathliquid or sheathless applications. In sheathliquid applications the ionization process is already aided by high concentrations of organic solvent in the sheath liquid making it unnecessary to add it to the BGE from an ionization standpoint. In sheathless applications the addition of an organic solvent to the BGE would seem very logical, but flow rates in most sheathless applications are already very low and therefore an organic component is not needed [41, 42]. It has been shown that separation of peptides can be improved by adding some organic solvent to the BGE, which mainly influences EOF strengths and thereby increases or decreases separation times and peak capacities [72, 73].

5 Applied Coatings

The coating of a separation capillary can be performed for a number of reasons depending on the type of analyte and requirements for resolution and speed of an analysis. A thorough review of all coatings applied in CE and CE-MS can be found in two publications [74, 75]. In short, capillary coatings in bottom-up proteomics are used to reduce wall adherence of the large peptides that are left after tryptic digestion to the silanols on unmodified fused silica. A coating agent is therefore used to alter the charge state on the capillary wall to cationic or neutral.

In the case of CE-ESI-MS, it is not common to use dynamic coatings although a

combination of coaxial sheathliquid sprayer and a number of dynamic procedures have been reported [18, 56, 70, 76].

Covalently bound cationic coatings have been applied in both coaxial sheathliquid and porous sheathless interfacing applications. Positive coatings efficiently counter wall adherence effects and result in very efficient separations with high plate numbers. Positive coatings reverse the EOF direction which requires the user to apply reversed polarity (negative charge on the inlet) as CE separation mode. Consequently most peptides will migrate opposite of the EOF direction requiring the EOF flowrate to be higher than the electrophoretic mobility of the peptides in solution. A drawback of positively coating in a separation capillary is the significantly higher flow rate it will generate, especially at low BGE pH. Depending on the application these higher flow rates will significantly reduce the separation power (peak capacity) of a system. Pattky and Huhn recently proposed the use of a cationic coating, OHNOON, that produces a less powerful EOF than the regularly used coating procedures and thereby improving the separation power of the system [56]

By far the most impressive results for bottom-up proteomics using CE-ESI-MS are produced using capillaries that are neutrally coated using polyacrylamide. The covalently bonded polyacrylamide coating strongly reduces the capillary surface charge and thereby diminishing the EOF to negligible levels [74]. As CE does not need a linear flow rate in the system to produce a separation (as opposed to liquid chromatography), most efficient separations are obtained at minimal flow rate. As the porous sheathless interface requires a flow rate in the separation system to provide a stable ESI spray, a small (0.5 to 2 psi) pressure is applied on the capillary [41, 47]. Busnel et al. showed that using this coating, peak capacities of more than 320 could be obtained while maintaining stable electrospray at 4.5 nl/min [41]. In a direct comparison of both cationic and neutral coatings Sarg et al. found the polyacrylamide neutral coating far superior in the detection of PTMs on peptides obtained from rat histones [47]. In applications using the electrokinetic junction interface, polyacrylamide coating was mostly used to obtain the stagnant separation conditions required for cIEF [34-36], but was also used to optimize separation conditions in the analysis of a small quantity of E. coli [28, 77].

6 Sample loading

In classical CZE separation only 0.5 to 2% of the total capillary volume is used to load sample before initiation of separation. Depending on the internal diameter and length

of the capillary the volume of this sample plug can vary but will never be a 'significant' total volume. This poor loadability of a classical CZE separation system results in low sample concentration sensitivities and a significant proportion of the sample that remains unused. There are a number of approaches that have been explored to resolve the inherent poor loadability of a CZE separation system which can be divided in in-solution stacking and solid phase extraction procedures.

6.1 In-solution stacking

The most commonly applied approach for in-solution large volume injection (in bottom-up proteomics) is so called transient-isotachophoresis (t-ITP) [78]. In this approach a leading (very high mobility) and terminating electrolyte (very low or reversed mobility) are added to the sample. Upon application of the separation voltage the electrolytes produce a highly conductive zone which will make the analytes with mobility between that of the leading and terminating electrolyte concentrate into high concentration zones. After dissipation of the leading electrolyte the normal CZE separation can commence. Optimizing the injection volume and sample electrolyte concentrations are crucial to get sufficient stacking of all analytes (peptides) of interest [45].

A second less commonly applied approach for free solution stacking is pH mediated stacking. In pH mediated stacking a plug of high pH buffer is injected before the sample plug which then forms a barrier where, by deprotonation, the mobility of the analytes is restricted. As a result all analytes with a positive electrophoretic mobility will stack against this plug of low pH buffer before the plug dissipates as a result of the CE process and normal CZE follows similarly to the t-ITP process. The most recent example of this approach used in CE-ESI-MS bottom-up proteomics was by Dong et al. who used it to inject larger volumes of synthetic peptides that contained PTMs [14].

Another free solution stacking approach that can be used to achieve unprecedented pre-concentration factors is electrokinetic supercharging (EKS) [79]. It is essentially an extention of t-ITP or pH mediated stacking where the inlet of the capillary is placed in the sample while ramping the (loading) voltages. A drawback of this approach is that the sample is not passively sampled, meaning that the remaining sample is altered and a second analysis from the same sample vial will be different. The application of EKS also results in disproportionate sampling of the analytes by over-sampling the highly mobile analytes and relative under-sampling of the low mobility analytes. A common drawback of all described stacking procedures is that a gain in loadability is achieved at

expense of the separation power, as a large portion of the separation capillary is used to load the sample. Complex biological samples such as proteolytic digests usually contain analytes with a large variety in electrophoretic mobilities and as a consequences there will inevitably be better stacking (sharper peaks) for high mobility analytes and less stacking (broad or square peaks) for the low mobility analytes.

6.2 Solid phase extraction

The most common approach for solid phase concentration procedures for CZE is the incorporation of a SPE column into the separation capillary, which has been thoroughly reviewed in two recent publications [80, 81].Only two examples of in-line solid phase extraction have been published in recent years. Gimenez et al. used an immune affinity column to show the presence of a peptide resulting from recombinant erythropoietin [82]. A reversed phase SPE column was also used by Wang et al. for the pre concentration and in-line fractionation of a Pyrococcus furiosus tryptic digest [44]. Using consecutive elutions at increasing concentrations of organic solvent, the peptide and protein coverage could be significantly improved over straightforward injection of the sample. Figure 5-6 shows the schematic setup of the solid phase microextraction (SPME) coupled to the separation capillary and the resulting electropherogram.

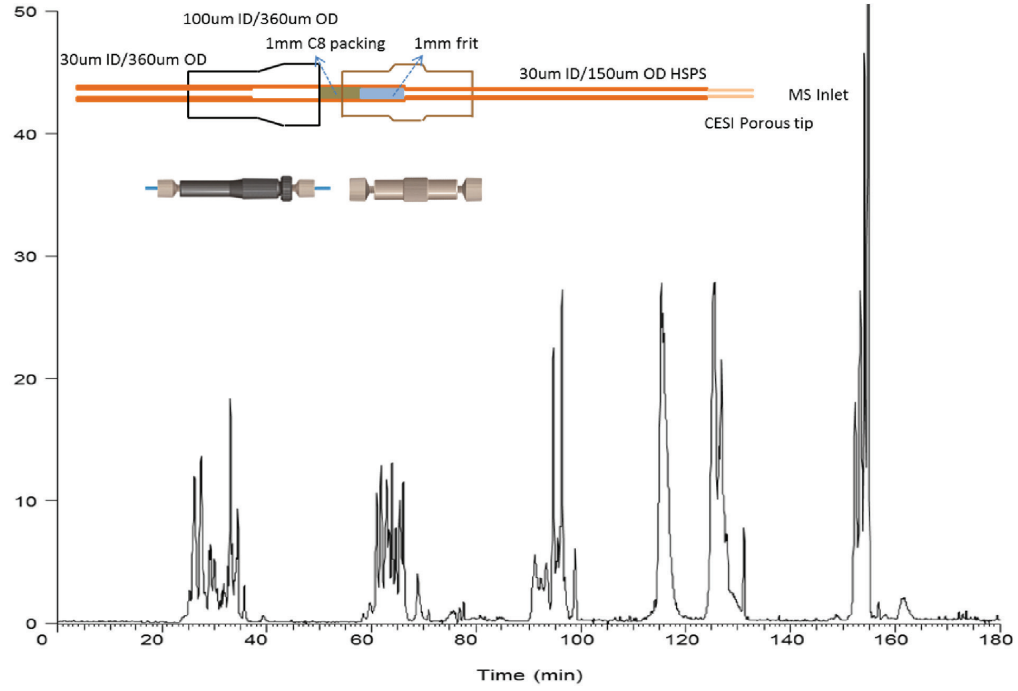

Figure 5-6: Schematic of the SPME-CE-MS/MS platform and base peak electropherogram for 5-step SPME multistep elution tITP-CE-MS/MS of 100 ng Pfu digest as published by Wang et al. [44]

6.3 Capillary Isoelectric Focusing

Although the combination of cIEF and ESI-MS is not a regularly applied technique, it is potentially the optimal method to overcome the poor loadibility of classical CZE separations [83]. Isoelectric focusing separates the analytes in the sample on basis of their isoelectric point. To achieve this separation almost the full capillary length can be filled with sample which needs to be pre mixed with an appropriate ampholyte. This ampholyte, together with the appropriate catholyte and anolyte creates a pH gradient over the length of the capillary. All analytes will become mobile in the system until they have reached the spot in the gradient corresponding to their iso-electric point resulting in concentrated bands of compounds that were previously evenly distributed through the whole sample.

Using cIEF in combination with ESI-MS is not straightforward process. It is for example impossible to perform cIEF-ESI-MS using a sheathless interface due to the requirement of a terminal catholyte at the capillary outlet. This catholyte is a high pH buffer which is not compatible with the positive ionization mode that normally applied for bottom-up proteomics. A number of recent publications show the applicability of cIEF-ESI-MS using the junction at the tip interfaces [34, 50]. Although a catholyte is very much needed for the cIEF process it causes a significant background signal as it is part of the sample solution that is sprayed during the ESI. As an alternative to the classic ampholytes Zhu et al. investigated the use of a 6 amino acid mixture as ampholyte [35] and found it to cause significantly less interference in the MS signals and provided sufficient focusing for the analysis of complex samples for semi-quantitative proteomics [36]. On the whole, cIEF-ESI-MS shows some great promise for larger scale applications but further development is needed before it will become a mainstream application in bottom-up proteomics.

7 Conclusions and outlook

With the rapid and ongoing development of mass spectrometers the use of high separation power in relatively short separation windows that can be provided by capillary electrophoresis is becoming increasingly more interesting for the analysis of highly complex samples. Although the drawbacks in CE-ESI-MS analysis are still numerous when compared to LC, the significant number of papers in the field of CE-ESI-MS proteomics shows that there is much and increasing interest.

With the development of interfacing strategies that allow for separation at minimal flow

rates in the separation capillary and highly sensitive ionization through nano sheathliquid and sheathless coupling, the options have been created to work on CE loadability which forms the last significant drawback.

It has been shown by the Yates group that the most significant drawback of CE, that of loadability, can be solved with some ingenuity and technical knowledge [44]. Although the use of in-line SPE also results in the loss of a portion of the peptides which would be problematic when whole protein coverage is desired, it is the most straightforward method for improving sample load capacity. The use of cIEF is a separate relatively simple approach to improve sample loadability, although application of this technique is not possible in sheathless approaches and deeper investigation is needed to find more suitable ampholytes [35].

A second issue is that of absolute separation power and peak capacity that can be achieved with the now commercially available CE systems. The use of neutrally coated separation and/or longer capillaries has pushed the peak capacities that can be obtained in capillary electrophoresis [41]. Nevertheless, the currently available systems were developed with applications in CE-UV and CE-LIF in mind which were generally applied to capillaries that were no longer than 30 or 40 cm. The simplest method to improve separation power is to lengthen the separation capillary with the unfortunate side effect that the potential/cm (which determines the separation power) diminishes. New generations of CE systems will need to be outfitted with stronger power supplies that will accommodate the longer capillaries of even up to 2 meters that are now being used for CE-ESI-MS bottom-up proteomics [44].

Finally, the potential of CE-ESI-MS in the more novel fields of proteomics, namely top and middle-down, is yet to be explored. Especially when compared to liquid chromatography the free liquid separation nature of capillary electrophoresis will allow for strong developments in this field.

Sample	BGE/ampholytes	Capillary/coating	Sheathliquid	Mass analyzer	Remarks	Ref.
Erythropoietin and biosimilars tryptic digest	20 mM Ammonium Acetate pH 7.0	-	3.3 µL/min 60/40 (v/v) 2-propanol /H2O 0.5% formic acid	IT	In-line immuno affinity SPE	[82]
BSA tryptic digest	30 mM Ammonium Acetate pH 3.0	N-Methylpolyvinylpyridium (positive coating)	2 µL/min 80/20 (v/v) MeOH/H2O 10mM acetic acid	TOF		[84]
Soy bean extract tryptic digest	0.5 M Formic Acid	-	3 µL/min 50/50 (v/v) 2-propanol /H2O	TOF		[85]
Recombinant human erythropoietin (rhEPO) tryptic digest	50 mM Formic Acid and 50 mM Acetic Acid	-	3.3 µL/min 50/50 2-propanol / H2O	IT		[86]
GST-lamin A/C (1–57) fusion protein tryptic digest	1-5 M Formic acid and 20% Acetonitrile	-	4 µL/min 50/50 2-propanol/ H2O 0.1% formic acid	IT	Large volume injection through pH mediated stacking	[14]
BSA Digest	750 mM Acetic Acid and 250 mM Formic acid or 150 mM Acetic Acid and 250 mM Formic Acid	Polyacrylamide (neutral coating), Polybrene and OHNOON (Positive coatings)	4 µL/min 50/50 2-propanol /H2O 1% acetic acid	QTOF		[56]
Immunoaffinity purified HSA carboxypeptidase A digest	100 mM Ammonium bicarbonate pH 7.8	Polyethylene oxide, Hydroxypropylcellulose (neutral coatings)	3 µL/min 60/40 acetonitrile/H2O 1% formic acid	IT		[70]
Human transferring tryptic digest	50 mM Formic Acid and 50 mM Acetic Acid	-	3.3 µL/min 50/50 2-propanol / H2O 0.05% of unknown acid	TOF		[87]

Table 5-1: Overview of coaxial CZE-ESI-MS proteomics applications reported between 2009 and 2013

Sample	BGE/ampholytes	Capillary/coating	Sheathliquid	Mass analyzer	Remarks	Ref.
Four bovine protein tryptic digest	10 mM ammonium acetate pH 7.0	-	50/50 MeOH/ H2O 10 mM acetic acid	Orbitrap		[88]
Insulin and β-Casein	10 mM ammonium acetate pH 7.0	-	50/50 MeOH/ H2O 10 mM acetic acid	Orbitrap	In-line protein trapping and digestion	[37]
Mycobacterium Marinum secretome tryptic digest	10 mM ammonium acetate pH 5.7	-	50/50 MeOH/ H2O 10 mM acetic acid	Orbitrap	RPLC prefractionated samples	[30]
BSA tryptic digest and added peptides	10 mM ammonium acetate pH 5.5	-	50/50 MeOH/ H2O 0.1% formic Acid	Qtrap		[32]
RAW264.7 cell lysate	10 mM ammonium bicarbonate pH 8.0	-	50/50 MeOH/ H2O 0.05% formic Acid	Orbitrap		[27]
Myoglobin, BSA, cytochrome c and Monoclonal antibodies	0.4% Pharmalyte (3-10)	Polyacrylamide (neutral coating)	50/50 MeOH/ H2O 0.05% formic Acid	Orbitrap	cIEF before mobilization and ESI	[34]
6 bovine protein mix and RAW264.7 cell tryptic digest	6 amino acid mix	Polyacrylamide (neutral coating)	50/50 MeOH/ H2O 0.1% formic Acid	Orbitrap	cIEF before mobilization and ESI	[35]
6 bovine protein mix and RAW264.7 cell tryptic digest	5 mM ammonium bicarbonate pH8.0	-	50/50 MeOH/ H2O 0.1% formic Acid	Qtrap		[31]
7 Protein mix and RAW264.7 cell tryptic digest	5 mM ammonium bicarbonate pH8.0	-	50/50 MeOH/ H2O 0.1% formic Acid	Orbitrap	In-line protein trapping and digestion	[38]
Escherichia Coli tryptic digest	0.1% Formic Acid	Polyacrylamide (neutral coating)	10/90 MeOH/ H2O 0.1% formic Acid	Orbitrap	Reversed phase SPE fractionated samples	[28]
CP12 cell tryptic digest	6 amino acid mix	Polyacrylamide (neutral coating)	10/90 MeOH/ H2O 0.1% formic Acid	Orbitrap	cIEF before mobilization and ESI of RPLC fractionated samples	[36]
α – Casein and BSA tryptic digest	Unknown basic pH buffer	-	Unknown	Orbitrap	2-dimensional separation with on-line alkaline phosphatase microreactor	[39]
BSA tryptic digest	Unknown basic pH buffer	-	Unknown	IT	2-dimensional separation with on-line alkaline phosphatase microreactor	[40]
Escherichia Coli tryptic digest	0.1% Formic Acid	-	10/90 MeOH/ H2O 0.1% formic Acid	Orbitrap		[77]

Table 5-2: Overview of kinetic nano-sheathliquid CZE-ESI-MS proteomics applications.

Sample	BGE/ampholytes	Capillary/coating	Mass analyzer	Remarks	Ref.
6 protein tryptic digest	0.1% Polybrene in 0.1% acetic acid	-	IT		[8]
BSA and Ecoli tryptic digest	10 % acetic acid pH 2.2	Uncoated and polyacrylamide (neutral coating)	TOF	Large volume injection through tITP	[41]
Histone H1 Endoproteinase Arg-C digest	0.1% Formic Acid pH 2.7	M7C4I, PolyE-323 and PEI (Positive coatings)	Orbitrap		[43]
Bovine milk tryptic digest	10% Acetic Acid pH 2.2	Polyacrylamide (neutral coating)	IT and TOF	Large volume injection through tITP	[42]
Pyrococcus furiosus tryptic digest	95 mM Acetic Acid and 5% MeOH	PEI (positive coating)	Orbitrap	In-line solid phase micro extraction	[44]
Antigen specific Immunoglobulin G tryptic digest	10% Acetic Acid	Polyacrylamide (neutral coating)	TOF	Large volume injection through tITP	[13]
Histone endoproteinase Arg-C digest	0.1%, 0.3% and 0.6% Formic acid, also 10% Acetic Acid	M7C4I and PEI (Positive coatings), polyacrylamide (neutral coating)	Orbitrap		[47]
BSA tryptic digest	90 mM Acetic Acid and 10% MeOH	Hydroxypropyl cellulose (neutral coating)	TQ	Spray needle coupled to large bore separation capillary	[45]
TrastuzumabTM (mAB) tryptic digest	10% Acetic Acid	-	QTOF		[46]
Yeast Enolase digest	0.1% Polybrene in 0.1% acetic acid	-	IT	Ultrafast CE in short and narrow bore capillary	[48]

Table 5-3: Overview of all porous sheathless CZE-ESI-MS proteomics applications

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Chapter 6

Workflow for Integrating CE-MS and LC-MS Bottom-up Proteomics Data from SDS-PAGE Pre-fractionated Samples

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Abstract

In recent years a number of studies have shown the strong complementarity of capillary electrophoresis and liquid chromatography for bottom-up proteomics analysis. The combined use of these strategies for more in-depth investigations has not found mainstream use due to two reason. The fractionation of samples before analysis by either CE-MS or LC-MS is currently performed to two separate techniques requiring larger amounts of sample and extra labor. SDS-PAGE followed by in-gel digestion as a fractionation technique is compatible with both CE-MS and LC-MS and therefore the same samples can be analyzed on the two platforms without much extra effort. Furthermore, the combination and comparison of data sets acquired by both analytical techniques is cumbersome an as of yet not automated. Here, a complete analytical workflow is presented for the fractionation, analysis and automated data processing of a sample analyzed by bottom-up proteomics. The combined analysis of a sample by both CE and LC-MS significantly improves the number of identified proteins and peptides and the developed data processing strategy reduces hands on time and provides an easy comparison and integration of the two data sets.

1 Introduction

The study of the proteins in a cells, tissues or other biological systems, which is the essence of proteomics, requires the analysis of highly complex biological samples. In the special case of bottom-up proteomics, enzymatic digestion increases the complexity of the original sample resulting in a large discrepancy between the theoretical peak capacity of one-dimensional separations and the required separation power. For this reason, many fractionation strategies to be applied before for both RPLC-MS and CE-MS analysis have been investigated. [1-5] The use of a pre-fractionation method, either on- or off-line, that is orthogonal to the second dimension separation based on hydrophobicity (reversed-phase liquid chromatography, RPLC) or charge and size (CE), increases the total separation power and results in increased peptide and protein identifications. In the case of pre-fractionation before CE-MS analysis, RPLC is most commonly employed in the first dimension.[6-8] While the most common pre-fractionation technique before RPLC-MS is strong cation exchange (SCX) chromatography. As the first and second dimensions in these strategies are nearly orthogonal; a significant increase in total peak capacity can be achieved resulting in a large increase in identified peptides when compared to a one-dimensional separation. A drawback to the two fractionation approaches described above (RPLC and SCX) is the loss of hydropylic and hydrophobic peptides for which CE-MS and RPLC-MS, respectively, are the ideal analytical strategies.

SDS-PAGE pre-fractionation is generally used before RPLC-MS, [9, 10] but with a small adjustment is also very suited for fractionation before CE-MS. The separations based on protein size and separation at the peptide level are fully orthogonal while retaining information on the identified proteins. Samples from SDS-PAGE, digested in-gel, are ready for analysis after evaporation and reconstitution in an appropriate sample buffer. Most importantly, peptides for which each separation method has most specificity are retained in SDS-PAGE fractionation. Here we show the use of a data processing workflow for combining and comparing CE-MS and RPLC-MS proteomics datasets obtained from analyzing an SDS-PAGE pre-fractionated sample.

The data processing workflow compares the two data sets on complementarity with regard to identified peptides and proteins and qualitatively for differences in hydrophobicity and peptide size. Furthermore, as the sample was pre-fractionated on one SDS-PAGE gel it is possible to perform a direct comparison of the identified proteins from the varying fractions. The developed workflow shows the identification of the proteins and compares

them to the fraction they were found in. This gives greater insight into the influence of the applied separation on the identification of proteins and peptides. The additional information obtained from the fraction to protein comparison can distinguish between proteins containing the same peptide, covalent protein-protein complexes[11] or large post-translational modifications, but will mainly show the presence of false protein identifications. For this investigation, a whole-cell protein extract from Escherichia coli was separated by SDS-PAGE in the first dimension, with subsequent tryptic in-gel digestion and analysis by both capillary RPLC- and tITP-CZE-MS/MS of the obtained digests. Although a comparison of two analyses of the same sample with different separation strategies is performed here, the developed workflow is a general tool and can be used in a broader sense to compare any two proteomics datasets that are obtained through SDS-PAGE pre-fractionated samples.

2 Materials and Methods

2.1 Chemicals

All chemicals used were of analytical reagent grade and obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) otherwise stated specially. All buffers and solutions were prepared in ultra-pure water from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated.

2.2 Sample preparation

E. coli cells were grown on LB medium (Life Technonogy™) washed with 1x gewassen 0.3 M Sucrose, hepes pH 7.0 and centrifuged into a pellet Protein extraction was performed using 50 µl of 1% SDS (containing protease inhibitor and 1 µl benzonase of 25 U/µl), placed at 4 oC for 30 minutes, centrifuged at 16,000g for at 4 oC for 15 min and subsequently the supernatant was taken. The protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit (Thermo Fischer Scientific) and 45 µg of protein was loaded on a 1 mm 10-well 4-12% NuPAGE® Bis-Tris gel (Invitrogen, Carlsbad, CA). Proteins were separated in the gel for 1 h at 180 V. The gel was stained in NuPAGE® Colloidal Blue (Invitrogen) overnight at room temperature and de-stained with milli-Q water until the background was transparent.The gel lanes containing the separated proteins were cut into 48 identical slices using a custom-made OneTouch Mount and Lane Picker (The Gel Company, San Francisco, CA). Each slice was placed in to a well in a 96-well polypropylene PCR plate (Greiner Bio-One, Frickenhausen

Germany). In gel digestion and peptide extraction were performed following a previously described protocol [12] with the adaptation that extraction of peptides from the gel was performed using acetic acid with a factor 10 higher concentration than the normally used trifluoroacetic acid. Consecutive sample wells were combined to obtain 24 samples and were split in two (± 15 µl each) as aliquots for CE and LC analysis.

2.3 Capillary Electrophoresis-Tandem Mass Spectrometry

All CE experiments were performed using a PA 800 plus capillary electrophoresis (CE) system from Beckman Coulter (Brea, CA, USA), which was equipped with a temperature controlled sample tray and a power supply able to deliver up to 30 kV. Separation capillaries with a porous sheathless interface to the mass spectrometer were provided by Beckman Coulter (Brea, CA, USA). Separation was performed on 90 cm long bare fused silica capillaries with 30 µm internal and 150 µm external diameter. The background electrolyte (BGE) and leading electrolyte (LE) consisted of 10% acetic acid (pH 2.2) and ammonium acetate (pH = 4 and 50 mM ionic strength), respectively. Injection volumes and flow rates were calculated using Poiseuille's equation and a fluid viscosity of 1.04 cP.

Preparation of the separation capillary and mass spectrometry interface end was performed as previously described.[13, 14] For the coupling of the sheathless CE sprayer to the mass spectrometer, a specially designed sprayer mount in combination with the Bruker nanospray shield was used. A stable ESI spray current was achieved at 1300 V.

The SDS-PAGE in-gel digests were evaporated to dryness and stored at -20 oC until reconstitution in 2 µl of 50 mM ammoniumacetate pH 4.0 before injection. Before every analysis the capillary was rinsed with 0.1M Sodium hydroxide (70 psi, 120 seconds), 0.1 M hydrochloric acid (70 psi, 120 seconds), water (70 psi, 180 seconds) and BGE (70 psi, 240 seconds) consecutively and the conductive liquid around the porous tip was refreshed (75 psi, 180 seconds). A total of 50 nl (2.5%) of the sample was injected through hydrodynamic pressure (6 psi for 60 seconds) followed by a plug of BGE (1 psi 25s). A 20kV separation voltage was employed for transient-Isotachophoresis (tITP), CZE separation and induction of the EOF (± 15 nl/min). The CE system was controlled by 32 karat from Beckman Coulter (Brea, CA, USA).

2.4 Liquid chromatography-tandem mass spectrometry

The LC-MS/MS analysis was performed using a splitless NanoLC-Ultra 2D plus system (Eksigent, Dublin, CA) with a 45-minute linear gradient increasing from 4% to 35% acetonitrile in 0.05% formic acid with a constant flow rate of 4 µL/minute. For each analysis, 10 µL of sample was loaded and desalted on a C18 PepMap 300 µm, 5 mm-i.d., 300 Å precolumn (Thermo Scientific) and separated by reversed-phase liquid chromatography using a 150 mm 0.3 mm–i.d. ChromXP C18CL, 120 Å column. 5 µl of ultra-pure water was added to each sample fraction.

2.5 Mass spectrometry

Mass spectrometry was performed on an amaZon speed ETD high-capacity 3D ion trap (Bruker Daltonics, Bremen, Germany) After each MS scan, up to ten abundant multiply charged species in the m/z 300-1300 range were automatically selected for MS/MS but excluded for one minute after being selected twice. The UHPLC system was controlled using the HyStar 3.4 with a plug-in from Eksigent and the amaZon ion trap by trapControl 7.0, all from Bruker.

2.6 Data analysis

We used Taverna Workflow Engine [15] to build a scientific workflow for the data analysis. The workflow combines general data processing steps for peptide and protein identification with the further statistical analysis on the all the files to combine and compare the fractionated data. All acquired tandem mass spectrometry data was processed using tools from Trans-Proteomic Pipeline (TPP)[16] embedded in the Taverna scientific workflow.[15] The raw data was converted to mzXML[17] using compassXport 3.0 (Bruker) and searched with X!Tandem.[16, 18] The X!Tandem output with peptide identifications and scores were then converted to pepXML,[16] and then processed using PeptideProphet to obtain the probability of each peptide-spectral match .[19] The X!Tandem search was here performed against the UniProt Escherichia coli reference set (2010-01-21) allowing a random error ±0.5 Da, +1 or +2 Da isotopic error, cysteine carbamidomethylation as fixed and methionine oxidation as variable modification and the k-score plug-in. (Parameter file included in supplementary material) After PeptideProphet mixture modeling and peptide-spectrum match probability estimation, resulting lists of peptide/protein identifications with minimum probability of 0.99 were analyzed and compared using statistical components in the Taverna workflow.[15] For

each peptide sequence, GRAVY scores[20] and full-sequence molecular weights were calculated. We generated cumulative distributions and histograms of the GRAVY scores and peptide masses. For advanced comparison between the two datasets, we plotted the protein molecular weight against the fraction of the two methods using CE-MS and LC-MS. The complete workflow including the data that was used for the creation of our figures can be found at http://cpm.lumc.nl/yassene/integrating_cems_lcms/.

3 Results and Discussion

3.1 Peptide identification and characterization

SDS-PAGE pre-fractionation allows for fractionation of samples based on protein size followed by digestion, thereby producing samples that have minimal loss in certain subclasses of peptides which would have been lost in traditional fractionation strategies. Furthermore, the samples that are obtain from in-gel digestions are compatible with both LC and CE-MS analysis making it possible to perform highly complementary analysis of one sample set by two techniques. Here we chose to make the throughput of the two methods similar. (Materials and methods, Figure 6-5) The combination of datasets from the same sample to obtain a more comprehensive picture of a sample's proteome

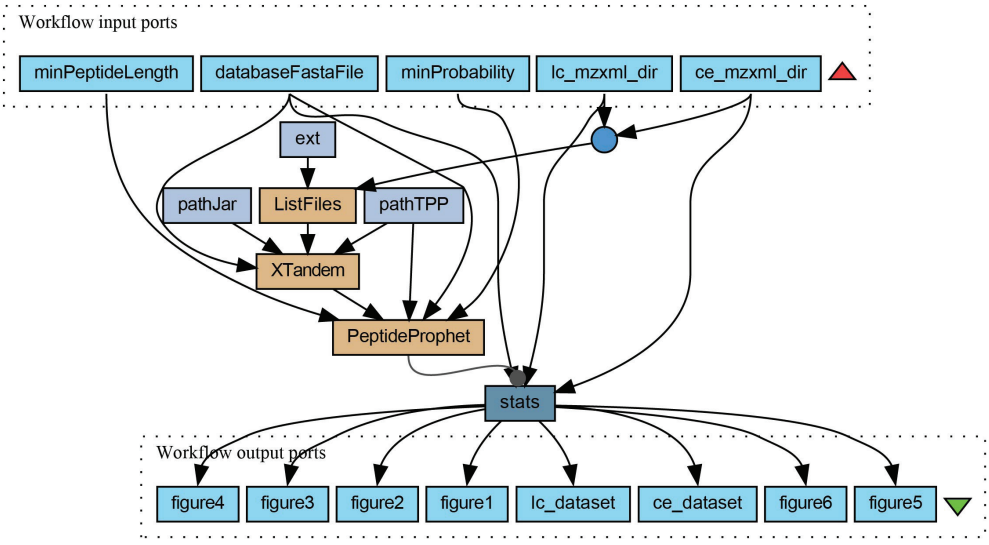

Figure 6-1: Taverna workflow developed for the data processing and comparison of the two proteomics data sets.

is becoming a more regular occurrence as not one strategy can show all peptides in a sample due to limitations with the separation technique. Our workflow performs a proteomics database search using X!Tandem followed by a qualitative comparison of identified peptides and proteins towards combining and comparing two sample sets obtained from two different or complementary proteomics methods, here LC and CE-MS. (Figure 6-1) The workflow accepts raw mzXML data and returns a set of figures and lists optimized for the most detailed description of the sample.

The output of the workflow consists of 5 figures each depicting a comparison of the two datasets on basis of a specific characteristic or output result. A Venn diagram shows the numbers of identified peptides and their overlap between the two data sets. (Figure 6-6) The numbers of confidently identified peptides between the two techniques is relatively similar with 2,846 peptides by CE-MS and 2469 peptides by LC-MS of which 1,363 could be found in both data sets. This means that there is a 60% complementarity in the identified peptides in CE-MS compared to the LC-MS data set.

The workflow is used to perform a more in-depth investigation of the characteristics of the identified proteins. The GRAVY score for all identified peptides was calculated and plotted as a cumulative distribution to portray which technique identifies more hydrophilic or more hydrophobic peptides. (Figure 6-2) Contrary to the observations made by Li

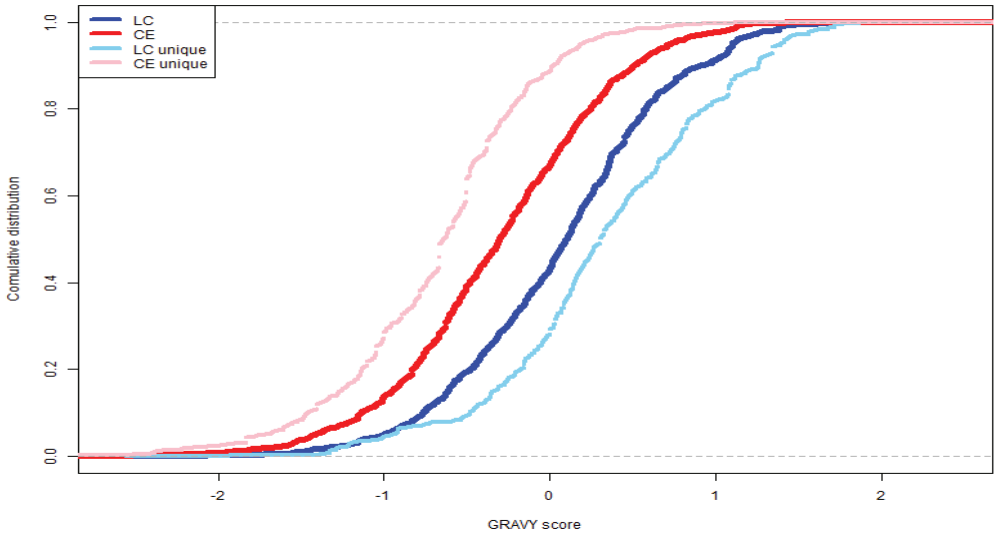

Figure 6-2: GRAVY scores are calculated for all identified peptides and plotted in a cumulative distribution. y axis represents all peptides in ascending order from low GRAVY score (y = 0) to the highest score (y = 1). The x axis represents the score corresponding to the peptide on the y axis. Methionine oxidation was not taken into account for this calculation.

et al. [6] there was a clear difference in average GRAVY score between tITP-CZE-MS and RPLC-MS for peptide identifications. Especially peptides unique to the individual technique show a large difference in average gravy score. The mass distribution of the identified peptides detected by the two respective techniques in Figure 6-3 does not show a big difference for the average mass of the identified peptides. It does show an increase in identified peptides in the 1,000 to 1,300 dalton range for LC-MS and for CE-MS in the 1,500 to 2,500 Dalton range. As the plots show absolute numbers of identified peptides and not relative amounts of the total peptides identifications the higher number of identified peptides by CE-MS was expected to give a slight deviation in the height of the curve. Nonetheless the curve shapes in Figure 6-3 are clearly different indicating that CE-MS was capable of identifying peptides with an average higher weight than LC-MS.

The difference in hydrophobicity and the discrepancies between the masses of the identified peptides give an explanation for the strong complementarity of CE-MS and LC-MS in the identifications of peptides from a complex sample. The identification of

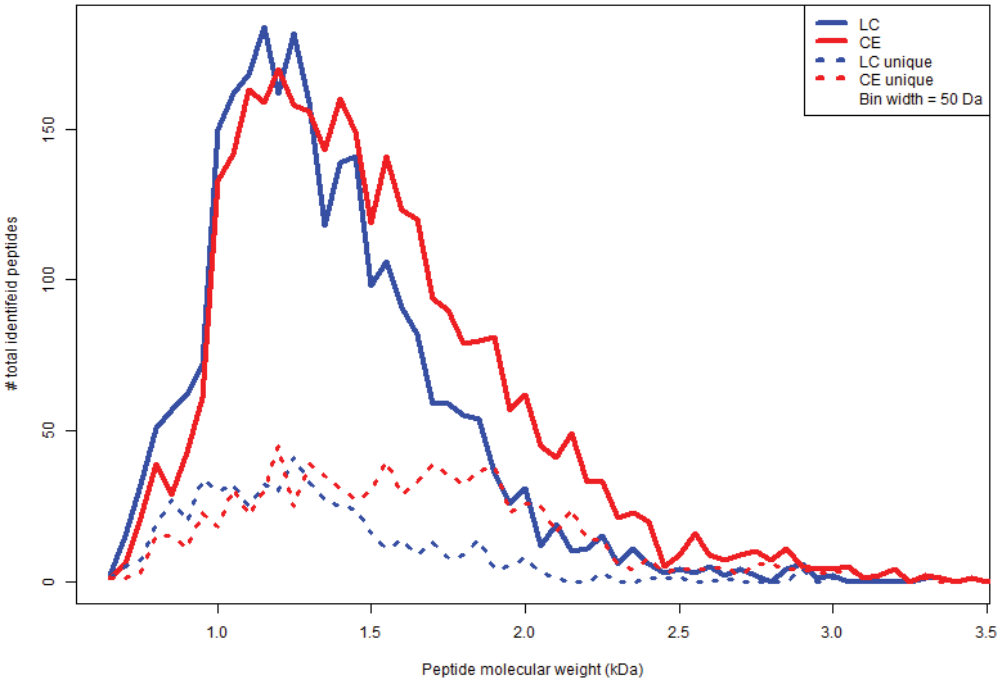

Figure 6-3: Distribution of the masses for the identified peptides calculated from the identified sequence for all peptides identified with a technique and unique identifications for either tITP-CZE (Red) or RPLC (Blue).

strongly varying peptides however is not by definition useful unless this also translates in complementarity in the identifications at the protein level.

3.2 Increasing confidence in protein identification

A second Venn diagram shows the numbers of identified proteins and their overlap between the two data sets (Figure 6-7). The identified peptides translates into 835 proteins identified by CE-MS and 811 by LC-MS with an overlap in identifications of 620 proteins. This means that the 60% complementarity in peptides translates in a 26.5% complementarity of CE-MS compared to the LC-MS data set on the protein level. When the identified protein masses are plotted against the SDS-PAGE fraction is was

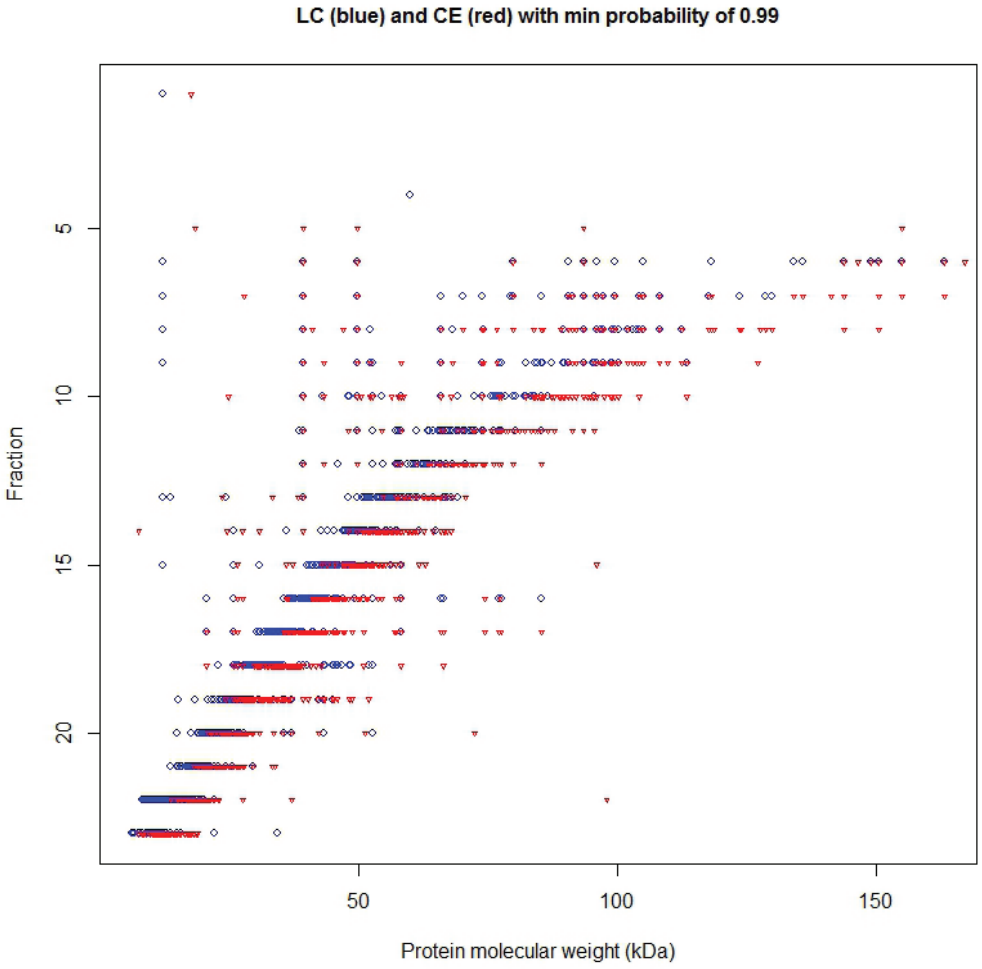

Figure 6-4: Distribution of the identified proteins found in the varying gel fractions by tITP-CZE-MS. Each protein is plotted only once corresponding to the fraction it was found with the highest confidence (protein score) The E. coli gel separation is shown (1) next to the molecular weight marker (2)

identified in there is no clear trend in either technique identifying proteins in higher or lower weight classes (Figure 6-7). An interesting phenomenon can be observed however in an overlapped plot of the two data sets. (Figure 6-4)

From Figure 6-4 it seems that RPLC-MS very consistently identifies lighter proteins in each fraction than those found by CE-MS. The explanation of this difference in identified protein mass could be found in the difference in the hydrophobicity of the identified peptides between the two techniques. Although SDS-PAGE assumes an average number of dodecyl sulfate molecules per kDa this is in reality a range which is dependent on the protein in question. Why this discrepancy between the masses of the identified proteins from the same fractions occurs warrants further investigation.

4 Conclusions

The analysis of one sample by two different analytical strategies has long been an interesting strategy to improve the proteome coverage of obtained from one specific sample. Although CE has been shown to be an excellent complementary separation technique to RPLC, earlier limitation of the technique which include sensitivity prevented the use of CE-MS as a consistent complementary technique to RPLC-MS. However, recent developments in the field of CE-ESI-MS have boosted its use in bottom-up proteomics and it was shown to provide excellent complementarity in identification of peptides.[21]

As SDS-PAGE fractionation is orthogonal to both RPLC and CE separations, it is ideal to perform in conjunction with either RPLC-MS or CE-MS, or with both of them and then combine the results for even larger coverage. Beside the actual laboratory work and data acquisition, the challenge in such an experiment is the amount of data collected that need to be processed in a standardized manner and then integrated to give an overall picture of the sample. The processing of big data sets consisting of a large number of files is cumbersome and the comparison of the two data sets requires a significant number of data manipulations before comprehendible results are obtained. The workflow presented here, was designed to process data obtained in such large scale experiments and produce simple and easy to read and comprehend figures for a quick overview of the results. In our test dataset we found that RPLC-MS and CE-MS are strongly complementary in the identification of peptides; most notably on basis of their hydrophobicity (GRAVY score) and also peptide size. Furthermore, there was a clear shift in the mass of the identified proteins in each fraction between CE-MS and

RPLC-MS. Although further investigation and tests are required to understand more the cause of this shift, it shows clearly that by combining the two methods we are obtaining a better coverage. Although we used the scientific workflow for the comparison of two analytical strategies applied on the same sample, the workflow is general in its purpose and can in principle be used for any comparison of two sets of SDS-PAGE fractionated samples. This can be the comparison of two different samples analyzed by the same second dimension strategy, comparison of different protein extraction strategies for the same sample, or even for investigating differences between biological or technical replicates.

5. Acknowledgement

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Supplementary Figures

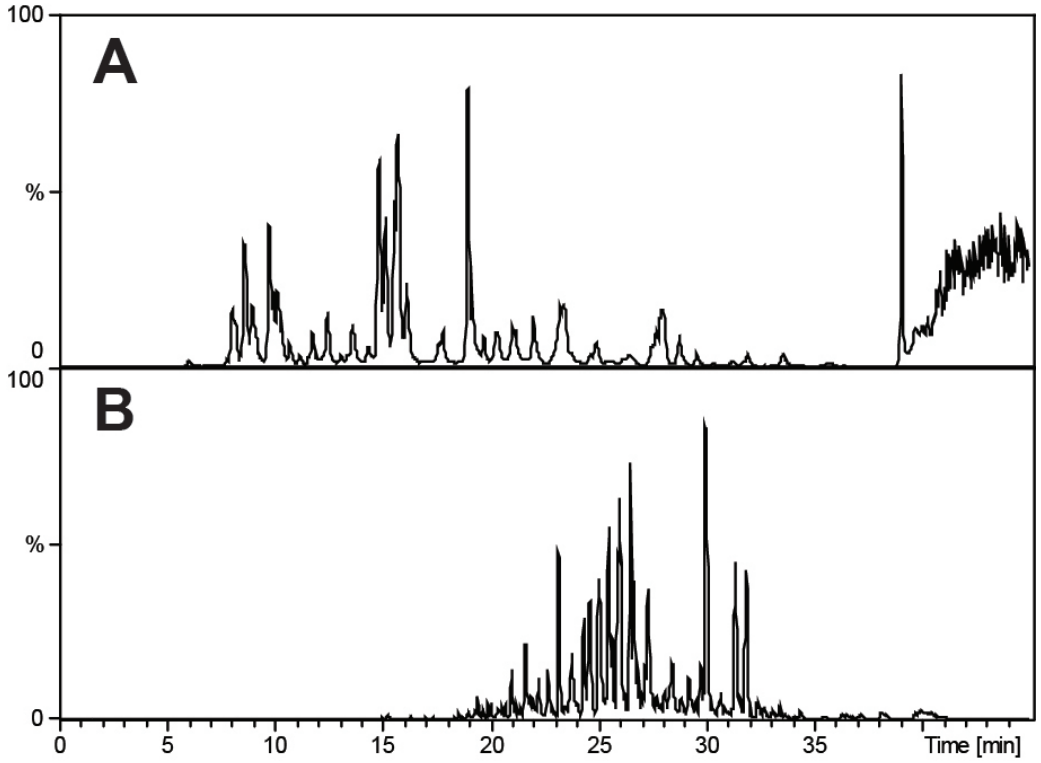

Figure 6-5: (A) Base peak Electropherogram and (B) Base Peak Chromatogram of the most abundant gel fraction (slice 12) obtained with the respective tITP-CZE- or RPLC-MS methods.

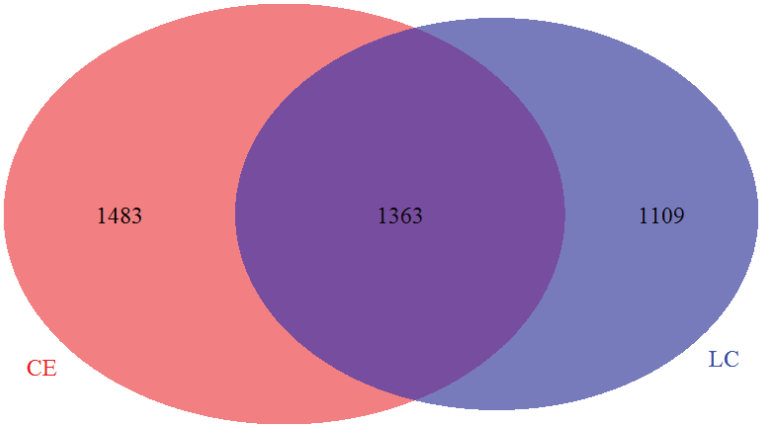

Figure 6-6: Venn diagram showing the overlap and the number of uniquely identified peptides in the two data sets

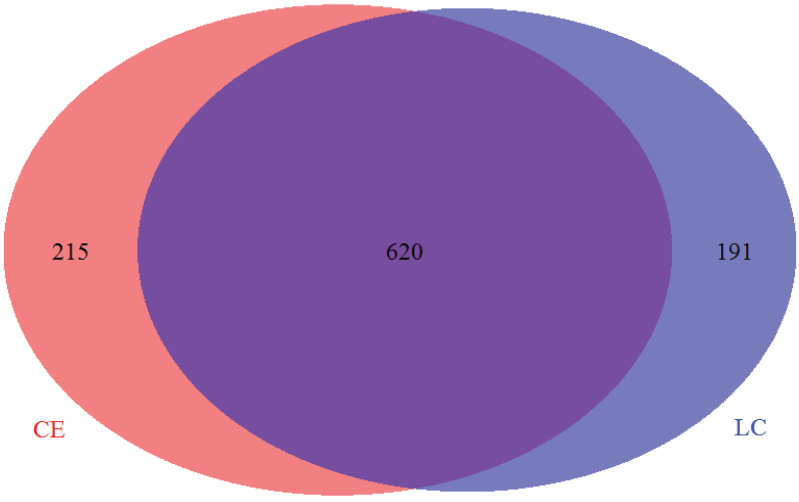

Figure 6-7: Venn diagram showing the overlap and the number of uniquely identified proteins in the two data sets.

Chapter 7

Proteomics Analysis of laser micro-dissected and sieve isolated Human Glomeruli from frozen tissue by t-ITP-CZE-MS

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Submitted for Publication

Abstract

The proteomic analysis of such minute amounts of sample requires specialized sample preparation and analysis technology. Both are not routine approaches in the today's analytical environment. In particular, the analysis of human glomeruli is a relatively unexplored field. Glomeruli are of interest because of their distinct morphology which often undergoes specific changes even at very early stages of disease development.

Here two approaches for the analysis of isolated kidney glomeruli are presented and the results discussed in detail. A large scale glomerular isolation followed by in-depth proteomics analysis through SDS-PAGE fractionation combined with CE-MS analysis provides additional knowledge on the observable proteome of human glomeruli. A total of 590 new unique proteins could be identified compared to the only other known in depth proteomic study on glomeruli.

The analysis of laser micro-dissected glomeruli shows the capability of determining dozens of proteins from even the smallest amounts of glomerular material. A sample preparation method allows for one-pot sample preparation before analysis by CE-MS. As the sample requirement of CE-MS is minimal, replicate analysis could significantly increase the number of identified peptides and proteins and resulted in the identification of over 100 proteins from material equivalent to only one human glomerulus.

1 Introduction

The glomerulus is a distinctive microscopic feature of the kidney. The morphological changes in the glomerulus provide an insight into the type and scale of the pathological processes within kidney tissue. However, as solid as morphological assessment of the pathology is, it provides no insight into the changes at the protein level. An understanding of the link between the proteome of a glomerulus and its morphology could provide insight into the pathological processes in the kidney. In-depth proteomics analysis by the Yamamoto group[1] on isolated glomeruli using large amounts of material identified 1817 non-redundant proteins. The authors provided a solid global overview of the glomerular proteome without links to any specific pathology.

With the development of laser micro-dissection (LMD) the harvesting of glomeruli presenting distinct pathology has been made possible. Nevertheless, the proteomics investigation of the varying pathologies has been limited, mostly due to a lack of a straightforward method for preparation and analysis of these very limited amounts of material. Until recently, only the groups of Rovin an Yamamoto have reported proteomics analysis of LMD human glomeruli. Both investigations isolated all glomeruli that could be found in a complete protocol needle biopt.[2, 3] The isolation of all glomeruli does provide increased sensitivity but the specificity of analyzing specifically diagnosed pathology is diluted out by also using material from non-affected glomeruli. To effectively analyze material showing specific pathology resulting in samples that can contain as little as one glomerulus, optimized samples preparation and analytical technology is required.

Recently, capillary electrophoresis - mass spectrometry (CE-MS) has shown to be specifically suited for sensitive proteomics analysis of mass limited samples due to its low sample requirement and ability to separated and be coupled to electrospray ionization at low flow rates resulting in high sensitivity.[4-6]

In the current manuscript we apply CE-MS to the analysis of glomeruli that were isolated from frozen human kidneys. Both to provide a baseline on the attainable number of peptides and proteins and to hopefully expand the known proteome of the human glomerulus, in-depth proteomics was performed on glomeruli that were obtained by a previously published sieving strategy.[7] The obtained data was compared to the data that was obtained by the Yamamoto group[1] to asses differences in the approach and identify any proteins that could be uniquely identified in the current approach.

Another point addressed in the manuscript is CE-MS tailored sample preparation. The published work on the analysis of LMD glomeruli was performed using liquid chromatography techniques.[2, 3] The sample preparations strategies that were used in these applications were not directly compatible with capillary electrophoresis. For that reason a new sample preparation method needed to be developed which minimized the sample handling and sample transfer which could result in loss of analytes. The developed technique uses a proven volatile chaotrope (2,2,2 trifluoroethanol (TFE)) for cell lysis and protein desolvation resulting in a sample that simply required evaporation and reconstitution before analysis by CE-MS.[8-12] Previously, TFE was used in proteomics analysis by LC-MS of varying types of samples including laser captured tissues, however the use of TFE has never found wide recognition as many standard protocols and kits for LC-MS proteomics are readily available. The field of CE-MS proteomics however is still in its infancy and therefore no kits developed specifically for CE-MS are available. This one-pot strategy was used for the identification of proteins from laser micro-dissected glomeruli corresponding with the amounts of material that would be obtained by the isolation of 5, 2, 1 ½ and ¼ glomerulus. For both in-depth and isolated glomeruli proteomics the identified proteins were evaluated for their added value to the knowledge of the glomerular proteome and potential for aiding in identification of an observed pathology.

2 Materials and Methods

2.1 Chemicals

All chemicals used were of analytical reagent grade and obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) otherwise stated specially. All buffers and solutions were prepared in ultra-pure water from Sigma-Aldrich (Zwijndrecht, The Netherlands)

2.2 Harvesting of human glomeruli from whole kidney

Tissue from a a 74 year old deceased female renal donor without renal disease was used for isolation of the glomeruli using a method described previously.[7] Her kidney was discarded by the transplant surgeon for mainly anatomical reasons. Before her demise, she had provided consent for use of her tissue for research. Briefly, cortical tissue was pressed with a flattened glass pestle through a metal sieve with 212 µm pore diameter, followed by a sieve with 150 µm pore diameter. The glomeruli were then rinsed from the surface of the 150 µm sieve and transferred to a tube using ice-cold phosphate-

buffered saline (PBS). The tube was then centrifuged for 1 minute at 1,200g to obtain a pellet. The supernatant was removed and the sample stored at -70°C. The pellets of frozen glomeruli were removed from the tubes and sections were taken and transferred to an Eppendorf tube and again stored at -70°C before SDS-PAGE pre-fractionation and in-gel digestion.

2.3 Harvesting of human glomeruli by laser capture microdissection

Frozen unfixed tissue was obtained from five kidneys of deceased renal donors without renal disease, who had consented to the use of their kidneys for research. The kidneys were histologically normal but nonetheless rejected by the kidney transplant surgeon for technical reasons. All kidneys were initially preserved by cold storage with UW solution (ViaSpan™). Tissue samples were stored in liquid nitrogen at -80°C. Frozen samples were cut into 10-µm sections using a Cryotome FSE cryostat. Sections were placed on membrane slides (MembraneSlide 1.0 PEN, Carl Zeiss Microscopy GmbH, Germany), air-dried for 1 hour and stored at -80°C. Before performing LMD, frozen-sections were again air-dried for 1 hour. LMD was performed with a Carl Zeiss Microscopy Palm Microbeam. (Hardware serial number MBC 01070, software: PALMRobo V 4.6.0.4.) The microscope objective was 20, its focus 7139-µm. Glomerular sections were photographed using a Zeiss camera Axio CAM IC. An equivalent of five full glomeruli (100 laser-dissected 10 µm glomerular sections per kidney) per ET kidney were dissected followed by 2, 1, ½, ¼ glomerular sections. Glomerular dissectants were collected in plastic adhesive Tube Caps (LOT 000762-13, Carl Zeis Microscopy GmbH, 37081, Göttingen, Germany). Frozen tissue was stored at -80°C.

2.3 SDS-PAGE prefractionation and in-gel digestion

Protein extraction was performed using 50 µl of 1% SDS (containing protease inhibitor and 1 µl benzonase of 25 U/µl), placed at 4 oC for 30 minutes, centrifuged at 16,000g for at 4 oC for 15 min and subsequently the supernatant was taken. The protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) and ± 30 µg of protein was loaded on a 1 mm 10-well 4-12% NuPAGE® Bis-Tris gel (Invitrogen, Carlsbad, CA). Proteins were separated in the gel for 1 h at 180 V. The gel was stained in NuPAGE® Colloidal Blue (Invitrogen) overnight at room temperature and de-stained with milli-Q water until the background was transparent. The gel lanes containing the separated proteins were cut into 46 identical slices using a custom-made OneTouch Mount and Lane Picker (The Gel Company, San Francisco, CA). Each

slice was placed in to a well in a 96-well polypropylene PCR plate (Greiner Bio-One, Frickenhausen Germany). The gel pieces were washed for 30 minutes in 70:30 25 mM ammonium bicarbonate:Acetonitriletwice followed by dehydration in 100% Acetonitrile for 10 minutes. Between all steps all fluid is removed from the wells, only leaving the gel. 75 µl of 10 mM dithiothreitol in 25 mM ammonium bicarbonate was placed in the wells and incubated at 56 oC for 10 min. The gel pieces were then dehydrated in 100% Acetonitrile for 10 minutes. 75 µl of 55 mM iodoacetamide was then placed in the wells and the gels were incubated in the dark at room temperature for 30 minutes. The gel pieces were then washed twice with 25 mM ammonium bicarbonate before dehydration in 100 % Acetonitrile for 10 minutes. After removing all liquid by both pipetting and vacuum drying in a sample concentrator (Eppendorf), 30 µl of 5 ng/µl porcine trypsin was added to each well and the samples were incubated at 37 oC overnight.

The reaction was quenched by addition of 1 µl of pure acetic acid and incubation at 37 oC for 1 hour. 20 µl of the sample was transferred to the capillary electrophoresis injection vial and 30 µl of 1% acetic acid was placed back in the sample wells with the gel pieces and again incubated at 37 oC for 1 hour. Finally all remaining liquid was transferred to its respective CE injection vial and all samples were evaporated to dryness in a sample concentrator. The sample was evaporated to dryness and stored at -20 oC until reconstitution in 2 µl of leading electrolyte buffer before analysis.

2.4 Single tube microscale sample prep

10 µl of 50 % TFE 50 mM ammoniumbicarbonate was placed into the cap of the vial in which the microdissected tissue sections were collected and stirred vigorously with the pipet tip. Content was spun down to the bottom of the vial and subjected to ultrasonication for 5 minutes. 2 µl of 20 mM dithiothreitol in 50 mM ammoniumbicarbonate was added and the vial was incubated at 60 oC for 30 minutes. Subsequently, 2 µl of 50 mM iodoacetamide in 50 mM ammonium bicarbonate was added and the vial was incubated at room temperature in the dark for 30 minutes. 10 µl of 50 mM ammoniumbicarbonate was added to the sample and subsequently placed in a Sample concentrator (Eppendorf) at room temperature for 20 minutes for a reduction of the TFE percentage. 1 µl of trypsin in 50 mM ammoniumbicarbonate was added to the sample in 100 ng/µl 40 ng/µl 20 ng/µl 10 ng/µl 10 ng/µl for the 5, 2, 1, 0.5 and 0.25 glomeruli samples respectively. The samples were incubated over night at 37 oC and the digestion was then stopped by adding 1 µl of pure acetic acid and incubation for 1 hour at 37 oC . The vial was then centrifuged at 16,000 G for 10 minutes and the sample was then transferred to the sample vial. The

sample was evaporated to dryness and stored at -20 oC until reconstitution in 2 µl of leading electrolyte buffer before analysis.

2. 5 Transient-isotachophoresis Capillary Zone Electrophoresis - Mass spectrometry

All CE experiments were performed using a PA 800 plus capillary electrophoresis (CE) system from Beckman Coulter (Brea, CA, USA), which was equipped with a temperature controlled sample tray and a power supply able to deliver up to 30 kV. Every analysis was preceded by a rigorous rinsing protocol consisting of 0.1 M NaOH, 0.1 M HCl and pure water consecutively before filling the capillary with BGE. Separation was performed at 20 kV resulting in an EOF of ± 15 nl/min in a capillary of 90 cm length and 30 µm i.d. and 150 µm o.d. The background electrolyte (BGE) and Leading Electrolyte buffer (LE) consisted of 10% acetic acid and ammonium acetate (pH = 4 and 50 mM ionic strength) respectively. Injection volumes were calculated to be 50 nl (11% capillary fill) using the Poiseuille equation, a fluid viscosity of 1.04 cP from a hydrodynamic injection of 5 psi for 70 seconds. All samples were reconstituted in 2 µl leading electrolyte buffer before analysis. Preparation of the separation capillary and mass spectrometry interface end was performed as previously described[6, 13].

For the coupling of the sheathless CE sprayer to the mass spectrometer, a specially designed sprayer mount in combination with the Bruker nano spray shield was used. Generally, stable spray for positive ionization was achieved between -1000 and -1300 V ESI Voltage, which was dependent on the distance between the sprayer tip and the MS entrance. Drying gas was set to 1.5 l/min (nitrogen) while the source temperature was set to 180 °C. Mass spectrometric analysis was performed using the UHR-QqTOF Impact HD system (Bruker Daltonics, Bremen, Germany).

2.6 Data analysis

Peak lists were generated from the raw spectra files using ESI Compass for amaZon 1.7 Data Analysis V4.2 SP4 (Bruker Daltonics, Bremen, Germany) and exported as Mascot Generic Files (MGF). These files were searched against the human protein database (Swissprot update of october 2014) using the Mascot search algorithm (Matrix Science) and Mascot software package 2.5. The Yamamoto group data was analyzed as a merged search of all files. The data of the laser capture micro dissection tissue was processed per sample while the data from the in-depth analyses was processed as a

merged data search. The parameters of the QTOF data search were: fixed modifications – carbamidomethyl (C) and variable modification – oxidation(M); trypsin missed cleavages – 2; MS tolerance (with # 13C=1) - 0.05 Da; MS/MS tolerance - 0.05 Da. A database search of the data obtained from the Yamamoto group[1, 14] was performed with similar criteria except that MS and MS/MS tolerances were set at 0.5 Dalton. The minimum requirement for a protein to be included was that it is determined by a rank 1 unique peptide with a peptide score above the identity threshold as determined by the 1% FDR. In the data from the laser micro-dissected tissue, there were often not enough decoy hits to determine the 1% FDR. Here a protein was included if it was identified by a rank 1 unique peptide with a peptide score above 25. Classification of the identified proteins was done using the PANTHER Classification system (www.pantherdb.org)

3 Results and Discussion

3.1 Transient-isotachophoresis capillary zone electrophoresis -mass spectrometry

High sensitivity and efficient separation are often presented as the key advantages of the CE-MS based methods. The advantages, however, are balanced by a significant drawback limited sample loadability. Transiens isotachophoresis (t-ITP) is the most straightforward method to improve CE-MS sample loading.[6, 15-17] As t-ITP can have a negative influence on the separation power of the system, depending on the loaded volume, careful considerations has to be made with regard to the loading amount and balance has to be found between loadability and separation power. Our experiments

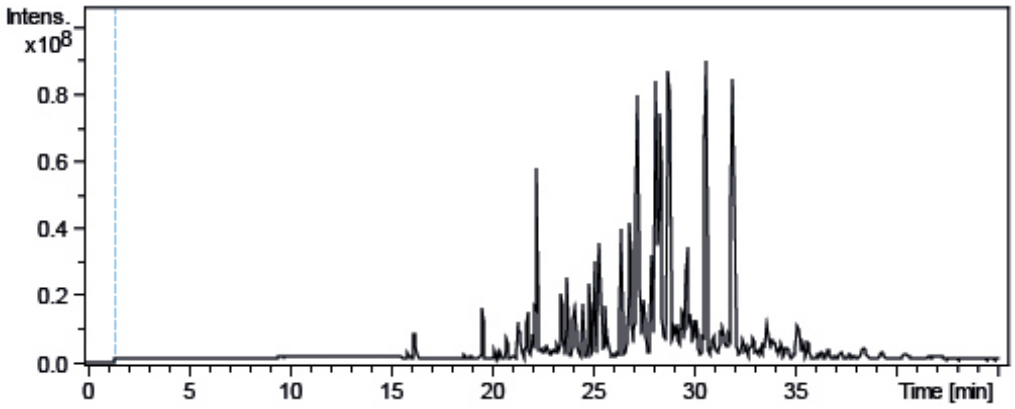

Figure 7-1: : Typical base peak electropherogram obtained from a 5 psi 70 second injection (± 50 nL and 8% capillary fill) and separation at 20 kV. This electropherogram was obtained from the analysis of in-gel digested fraction 32 of the total human glomerular SDS-PAGE gel.

(data not shown) show a sample load of 50 nl (± 8% of capillary) results in optimal loading/separation conditions for these experiments when using 50 mM ammonium acetate at pH 4.0 as leading electrolyte/sample buffer. Figure 7-1 shows a typical base peak electropherogram obtained using those conditions.

3.2 SDS-PAGE fractionated in-gel digested glomeruli

Although capillary electrophoresis has been used for the analysis of a number of pre-fractionated samples in recent years[18, 19] applying the technique for the analysis of a true complex tissue digest has yet to be performed. We have found that with a few minor adjustments a standard SDS-PAGE followed by in-gel digestion provides excellent pre-fractionation and results in samples that are optimally suited for capillary electrophoresis. After analysis of all 46 fractions a total of 1453 protiens and 7391 peptides were identified and a total of 895 proteins were identified with at least two unique peptides.

The Yamamoto group published the discovery of 6686 proteins from human glomeruli in 2007[14] but after re-analyzing the data and scanning for redundancy only 1817 proteins could be confidently identified.[1] The Yamamoto group used the international protein index database for their searches which has not been updated for a number of years. For this reason the raw data used for the Miyamoto et al.[14] and Cui et al.[1] publications was re-analyzed using the most recent Swissprot curated database for more confident identification and obtaining more easily comparable results. Using our criteria, 1324 unique non-redundant proteins could be identified from the Yamamoto group data and of these 863 proteins overlapped with the identifications from our data set. This means that we discovered 590 unique proteins in the CE-MS glomerular proteomics data set. Of the newly identified proteins 252 were highly confident identifications requiring two or more unique peptides per protein.

Using the Panther classification software tool (pantherdb.org) we found that there is very little difference in the identified proteins in the Yamamoto data set and ours with regards to the cellular component they are ascribed to. (Figure 7-2 A and B) A comparison of the unique proteins from our data set with the Miyamoto/Cui data set shows that a larger percentage of the identified proteins are ascribed to macromolecular and membrane origin. This is not surprising as the protein extraction method used to create our sample set utilized a surfactant (SDS) which would be more capable of extracting more hydrophobic (membrane) proteins and the less soluble macromolecules. The significance of these results is low however as only 47% of the proteins in the Miyamoto/

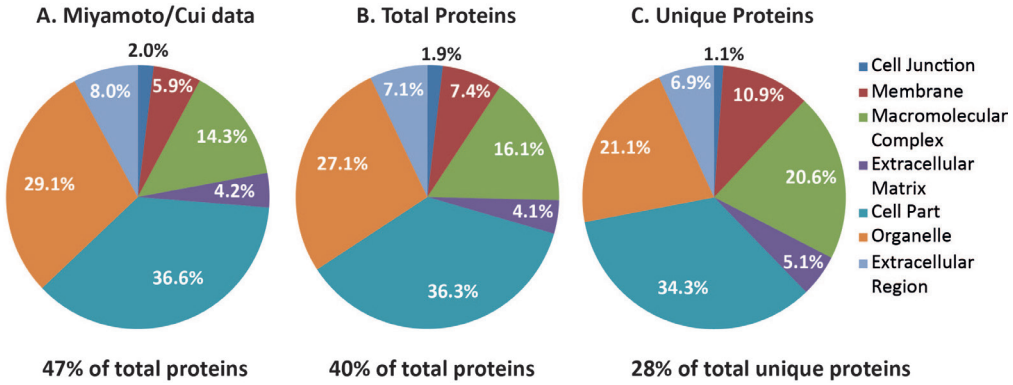

Figure 7-2: : Pie charts showing the subdivision of the identified proteins in their association with different cellular components. The classification was performed using the Panther classification software tool (pantherdb.org).

Cui data set and 40% of all proteins and only 28% of the unique proteins from our data set could be ascribed to a specific cellular component.

Although glomerular proteome shows significant overlap with that of other part of the kidney among the identified proteins there were 7 proteins in the list that are determined to be specific to the glomerulus only (Yellow in supplementary material protein list) Specifically, Nephrin and Podocin were identified which are very important in the functioning of the renal filtration system. While the Kin of IRRE-like protein 1 is very important in the development of glomerular permeability. Furthermore, Fibrinectin, Collagen type IV and Laminine are part of the glomerular basement membrane to which cells are bound with binding proteins of which Integrin could be identified specifically. These proteins are not necessarily specific to glomeruli to human glomeruli but do play an important role in its development and structure. The only glomerulus specific protein that was identified in our data set that was not present in the Miyamoto/Cui data set was Nck-associated protein which like Nephrin and Podocin is important in the function of the renal filtration system. Nck-associated protein was identified with very high confidence with a total of 5 unique peptides. Although few glomerulus specific could be additionally identified in our analysis the known glomerulare proteome was expanded by nearly 50% with a total of 590 proteins.

3.3 Analysis of Laser capture microdissected glomerular material

An average glomerulus in adults (3.9 µm3 ± 1.3 µm3), contains about 3600 cells per glomerulus and has an average diameter of about 200 µm which requires isolation by laser capture microdissection.[20] For an estimation of the influence of the amount of

material on the number of identified peptides and proteins using this approach, glomeruli were laser captured in a range of 5 sections (an equivalent of a quarter glomerulus) to 100 sections (equivalent of five glomeruli). Preparing such small amounts of material for proteomics analysis is very difficult and required the development of a specific one-pot sample preparation approach found in the Materials and Methods section. After analysis of the described samples the numbers of identified peptides and proteins some variation between samples (Table 7-1). One of the samples was analyzed in triplicate to also assess the variation in the number of identification between technical replicates. Figure 7-4 in the supplementary material shows the overlap op the identified proteins and peptides between technical replicates of the 5 glomerulus sample. As only 2.5% of the sample is injected for each analysis, performing replicate analyses of a precious sample is possible and will result in a significant increase in the number of identifications. Here the replicate analysis of the 5 glomeruli sample resulted in an increase of 50 % in the number of identified proteins and 70 % in the number of identified peptides compared to a single analysis of the very same sample. (Figure 7-4) For material equivalent to one glomerulus the triplicate analysis resulted in an addition of 80% extra identified proteins for a total of 117 proteins.

In all of the samples the proteins involved in cell and tissue structure were the most prevalent and therefore identified with highest significance. (Actin, Vimentin etc.). Despite the rinsing of venous system of the selected kidneys before the storage Albumin and some other blood related proteins can be found in varying concentrations. Besides the structural proteins we could also consistently identify Laminin and Collagen type IV from even the smallest amounts of material (5 sections) and with increasing amounts of sample (10 and 20 sections) Fibronectin and integrins were more consistently identified. Glomerulus specific proteins can be identified in even the smallest amounts of material (podocalyxin) with more being identified in higher amounts of material.

	Number of sections				
N = 5 biological replicates	**5**	**10**	**20**	**40**	**100**
peptides	118 ± 41	251 ± 66	324 ± 71	566 ± 64	578 ± 144
Proteins	32 ± 8	70 ± 25	86 ± 19	139 ± 26	136 ± 20
N = 3 technical replicates					
Peptides	68 ± 4	236 ± 7	259 ± 21	454 ± 6	385 ± 21
Proteins	26 ± 2	57 ± 3	73 ± 7	128 ± 5	112 ± 5

Table 7-1: Peptides and proteins identified from 5 biological replicates and triplicate analysis of one of the samples.

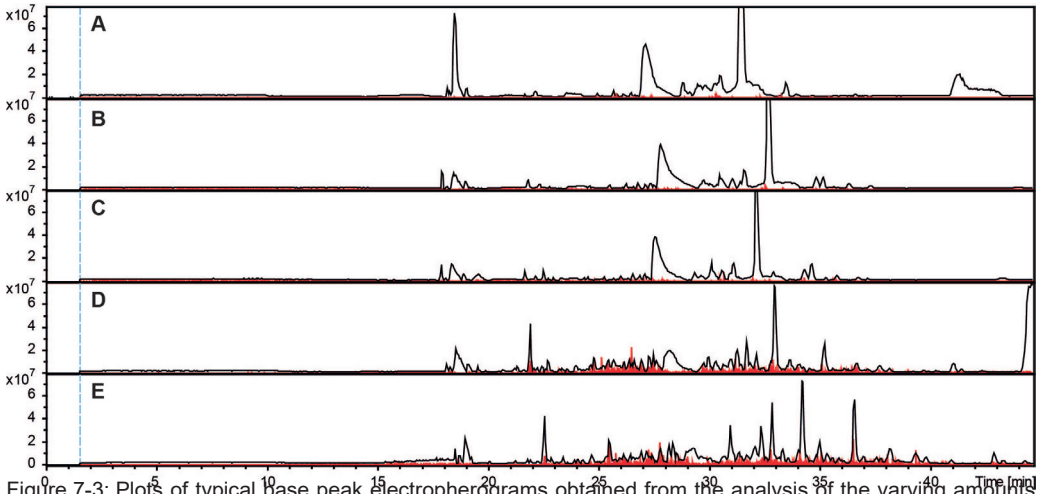

Figure 7-3: Plots of typical base peak electropherograms obtained from the analysis of the varying amounts of laser captured glomerular material. (A) 5 sections, 0.25 glomerulus (B) 10 sections, 0.5 glomerulus (C) 20 sections, 1 glomerulus (D) 40 sections, 2 glomeruli (E) 100 sections, 5 glomeruli. Data was not aligned resulting in small shifts in migration time between analyses.

Typical base peak electropherograms of the samples with varying amounts of material are depicted in Figure 7-3. The analyses were performed on unmodified silica capillaries and the data was not aligned after analysis. Therefore, some sample dependent migration time shifts are to be expected. On the whole we did not find the variation in migration time to be significant enough to strongly influence the peak capacity and identification power of the system. As the laser captured tissue is caught in the cap of a vial which contains a layer of an adhesive substance it was to be expected that some interference would be observed in analysis. In this case one large peak in the middle of the electropherogram is observed which unfortunately causes some ion-suppression resulting in poor identification of peptides in that migration window. We assume this peak is collection vial related as the analysis of cell digestions that were performed in Eppendorf tubes did not yield such interference.[12]

Conclusions

In this research we've shown the potential of CE-ESI-MS for bottom-up proteomics analysis. A previously used pre-fractionation strategy was adjusted to provide optimal sample quality for analysis by t-ITP-CZE-ESI-MS. We found that pre-fractionation by SDS-PAGE followed by in-gel digestion provided excellent samples which retains the smaller and/or more hydrophilic peptides which would have been lost in a fractionation strategy employing HPLC. From a total of ± 30 µg material from isolated human glomeruli we were able to identify 1453 proteins and well over 7000 peptides from those proteins. Especially the very rich gel fractions showed that more separation power through higher

peak capacity would most likely provide higher numbers of identified peptides and proteins, but despite the relatively low loadability (2.5 % of samples), sensitivity did not seem to be a limiting factor.

The analysis of laser captured micro dissected human glomeruli tissue was successful. There was a clear trend in the identification of the number of peptides and proteins showing that we are not yet restricted by the limited separation window that is observed using the current CE-ESI-MS setup. We consistently found the most important building blocks of human glomeruli to be the most significant protein identifications. This shows the current protocol will be able to identify proteins from glomeruli showing visible pathology as the observed changes are most likely significant on the protein level.

Although the current method is very capable in the analysis of both complex as well as depleted samples some development might improve the obtained results. Firstly, the use of longer or neutrally coated capillaries will result in longer separation windows providing improved identification capacity. Furthermore, larger sample volumes can be injecting using these setup which will improve sensitivity.

Acknowledgements

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Supplementary Figures

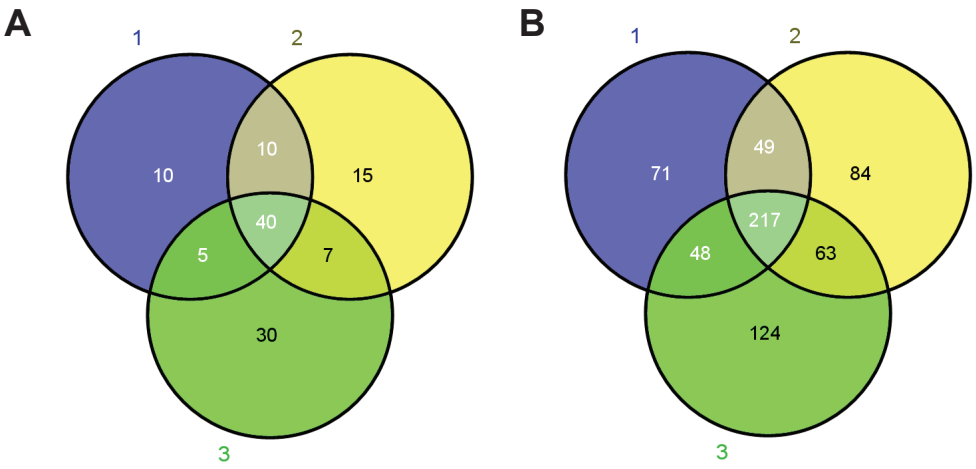

Figure 7-4: The proteins identified from three replicate analysis of a 20 sections (1 glomerulus) (A) and 100 sections (5 glomeruli) (B) sample.

Discussions and Conclusion

Progress in porous sheathless CE-ESI-MS

The development of electrospray ionization has transformed mass spectrometry into a technique that can now be found in almost every lab performing biological analyses. The technique has further matured over the years making it possible to analyze compounds of increasingly larger mass and also with increasingly higher sensitivity. It was found that decreasing the flow rates of the solvent containing the target analyte increased sensitivity and nano-liquid chromatography has optimally utilized this feature to become the dominant approach in the analysis of peptide mixtures and protein digests. Electrospray ionization at ultra-low flow rates has shown to have significant advantages over conventional nano flow rates (±300 nl/min) for not only sensitivity but it also minimizes ionization efficiency bias for specific compounds. The porous sheathless interface has shown to consistently generate a stable electrospray at flow rates below 10 nl/min and could therefore potentially produce the same ionization effects that have previously been observed with regards to reduced ion suppression[1]. **Chapter 1** investigates the potential of the porous sheathless interface at ultra-low flow rates for reduction of ion suppression in the ionization phosphorylated peptides. For these experiments five synthetic peptides with a constant amino acid sequence and varying numbers of phosphorylations (0-4) were obtained. A solution containing all 5 peptides was infused at flow rates varying from over 100 nl/min to only a few nl/min. The effect of reducing the electrospray flow rate was a significant increase in sensitivity for the multiply phosphorylated peptides with the greatest improvement (factor 4) for the peptide containing 4 phosphorylations. When the reduced electrospray flow rates were employed in a CE-ESI-MS analysis of bovine milk digest we observed an increase in identified phosphopeptides in a comparison with nano-LC-MS analysis of the same sample. A number of multiply phosphorylated peptides were among those identified using CE-ESI-MS where only mono phosphorylated species were found using the nano-LC-MS approach.

The ionization efficiency of the interface was also shown for the analysis of IgG 1 derived glycopeptides. Using a neutrally coated capillary the separation could be performed at flow rates below 10 nl/min and therefore large volume injection through transient isotachophoresis combined with ultra-low flow ionization resulted in a 20 fold improvement in sensitivity compared to the currently used platform nano-LC-MS platform[2] **(Chapter 2)**. While the standard platform was dedicated to high throughput screening, the CE-ESI-MS was used as a complementary approach for those cases where lack of sensitivity was evident. In this way we secured the optimal use of the

valuable clinical samples. The results from this investigation also showed that CE-MS has immense potential for identification of novel glycosylated peptides as CE separation very specifically separates the glycopeptides on basis of the present glycan moiety which for identification purposes could be easily exploited.

The separation at ultra-low flow rates is not only beneficial for the ionization process but equally important are the significant improvements in separation power that can be achieved using this approach. Unlike liquid chromatography, capillary electrophoresis does not require a linear flow in the separation system for effective separation. Through the mechanism of a porous silica section the porous sheathless interface does not require a linear flow in the separation system to maintain a closed circuit contact at the cathodic end of the separation system. This allows for separation at insignificant flow rates in the separation system while still using detection by mass spectrometry as soon as optimal separation is obtained. **Chapter 3** shows the potential of a period of Zero-Flow separation combined with large volume injection before eventual mass spectrometric. It was found that 100% increase in peak capacity could be achieved using this approach with minimal deterioration of peak shape therefore maintaining sensitivity. Such improvements in peak capacity can directly translate in increases in numbers of identified peptides in bottom-up proteomics of a complex sample.

CE-ESI-MS bottom-up proteomics

Although CE-MS does not belong to the mainstream methods used in bottom-up proteomics, a number of groups have explored CE as an alternative to the current standard nano-LC-MS. To obtain a perspective of the history and applications in the field of CE-MS proteomics and more specifically CE-ESI-MS bottom-up proteomics two in depth literature reviews were performed. The first review **(Chapter 4)** looks at the field of CE-MS bottom-up proteomics as a whole and the developments made in recent years. This review covers both CE-MALDI and CE-ESI-MS including the varying interfaces that are available for this technique. It shows that although CE-MALDI is still being used it a number of labs the general trend is away from this combination. CE-ESI-MS hyphenation strategies were shown to be less cumbersome and more robust and sensitive. The application of CE-ESI-MS in bottom-up proteomics showed to be very promising although very few publications on truly complex samples had been published at the time thus few conclusions could be drawn. The low sample requirement of CE-ESI-MS, however, was one of the features which makes it interesting for the analysis of very material limited samples, with ultimately the goal of single cell proteomics. For this, the development of

an improved injection protocol or of equipment which allows for injection from only a few hundred nano-liters would be required.

The review in **Chapter 4** showed the promise of CE-ESI-MS for bottom-up proteomics but it also showed it requires the development of optimal sample loading, separation and ionization conditions before mass spectrometric analysis. A critical look at all the required conditions in such a separation and analysis setup resulted in the review presented in **Chapter 5**. In this review each aspect of the separation system is discussed separately and placed in the larger context of developed applications to provide a novel user of CE-ESI-MS a frame of reference to start with. The review shows that the use of low conductivity and low pH buffers is very prevalent which is not surprising as buffers with high conductivity are often not compatible with CE-ESI-MS or would result in cluster formation during ionization as they regularly contain varying salt types and concentrations. There are three interface designs that have presented themselves as alternatives to the co-axial interface design that has been the dominant approach ever since its introduction in the late 80's. Of the three designs the electrokinetic junction interface has been used in the highest number of publications in the bottom-up proteomics field but only a few by groups outside the lab that developed this interface. The porous sheathless interface is the approach used by the largest number of groups and seems to be the easiest to adopt into a lab environment since becoming commercially available in 2014. The strongest drawback to CE and therefore CE-MS is the lack of loadability. A number of papers have shown that the new CE-ESI-MS interfaces can achieve high sensitivities from only small amounts of sample but the analysis of very depleted samples is still very difficult as only a small percentage of the sample can be injected. The solution to this problem can be found through two ways or even a combination of both; firstly larger volume injections through in-line SPE or stacking procedures can give much higher sensitivity, secondly mechanical improvements need to be made to CE injection systems to allow for injection from increasingly smaller sample volumes. As the mechanical improvement of CE systems is outside the capability of most analytical laboratories, the use of large volume injection through stacking and SPE are by far the most explored avenues. SPE-CE however, is very difficult to use and issues with reproducibility are a regular occurrence because already small changes in back pressure from the SPE column can alter the EOF. Column to column reproducibility is therefore an increasingly important issue as production on such small scale cannot currently be achieved with the required standardization. For this reason large volume injection was the method of choice to improve sensitivity in all chapters of this thesis.

At present the standard approach for separation in a bottom-up proteomics workflow is the use of a (nano)-reversed phase liquid chromatography (RPLC) system. Claims have been made that CE-MS is an excellent complementary technique to RPLC as it separates more specifically for peptides that are highly hydrophilic, which would most likely be lost in the solvent void of the RPLC separation. For in-depth proteomics approaches, however, RPLC is commonly used as a first dimension fractionation technique before CE-MS analysis. The result of this is that the fractionation approach already loses the peptides that were meant to be gained by CE-MS analysis. **Chapter 6** describes the development of a data processing workflow for the comparison of samples that are fractionated by SDS-PAGE. Because SDS-PAGE separates on basis of protein size/ mass it is completely orthogonal to RPLC and CE separations making the fractions suitable to be analyzed by both. A comparison of the two analyses would then show to what extent the two techniques are complementary in bottom-up up proteomics of more complex samples. The developed workflow showed excellent complementarity between the two strategies on both the identified peptide numbers and their hydrophobicity and peptide size. Moreover the data set from CE-MS identified more peptides and proteins.

Shotgun proteomics of minute sample amounts is an endeavor that is both challenging and potentially extremely rewarding. The rapid developments in the fields of sample processing (laser microdissection) and analysis (miniaturized machines) have resulted in major improvements in the “specificity” of the obtained sample and the sensitivity of its analysis. The development of the porous sheathless sprayer has made the low sample requirement of CE and excellent sensitivity of ultra-low flow electrospray available to the field of proteomics analysis. As the porous sheathless interface is only a recent development[3], this thesis has described the progress that was made in the application of the porous sheathless interface aiming at using it in CE-MS proteomics analysis of minute sample amounts.

The experiments in **Chapter 7** show the application of the sheathless CE-ESI-MS platform to the analysis of human glomeruli. A strategy similar to the one used in **Chapter 6** was applied to obtain in-depth proteomics knowledge on human glomeruli. Compared to the only previous in-depth experiment on human glomeruli, significantly more proteins were identified by our CE-MS platform. The analysis of laser micro dissected glomeruli from protocol needle biopsies is the ultimate goal in proteomics analysis of glomeruli. The analysis of such a sample was previously only performed on all isolatable glomeruli from a complete biopsy. While this does provide more material to analyze, the effect of analyzing one specific glomerulus that presents pathology is diluted by the unaffected

tissue. An investigation was performed by using isolated glomeruli from cadaver kidneys as a model sample to determine the number of proteins that can be identified from a certain amount of material. It was found that, using the developed strategy material, equivalent to only one glomerulus is enough to identify more than 100 proteins. The CE-MS method has very low sample consumption and therefore replicate analyses are possible and at n=3 an increase of 80% in identified proteins could be achieved. Even in the case of triplicate analysis only 7.5% of the sample was used making it possible to potentially perform complementary RPLC-MS analysis on the same sample to increase the number of identified proteins even further.

Conclusions and future prospects

The development of the porous sheathless interface for CE-ESI-MS offered a practical solution for a problem that had been plaguing the CE-MS community since the first coupling of CE with MS. This interface allows for highly sensitive and stable electrospray without dilution effects of the sheathliquid strongly reducing analytical sensitivity. The experiments in the first three sections of this thesis **(Chapters 1-3)** show that the developed sprayer results in a highly versatile technique that can be applied to a range of problems. The subsequent application of the technique in bottom-up proteomics shows that it even holds its own in a field that predominantly uses nano-liquid chromatography.

The future of this technique needs to be split in potential expansion of the fields of applications and also potential developments that can make this technique even more versatile and wider applicable. **Chapter 2** clearly showed the potential of sheathless CE-ESI-MS in the analysis of glycosylated peptides which are generally so hydrophilic that they are hard to analyze by conventional RPLC-MS approaches. Although the detection of glycosylated peptides combined with peptide mapping has occurred using this technique, targeted profiling or quantitative analysis of glycosylated species using this technique has not been performed except for the study in **Chapter 2**[4]. The field of intact protein analysis would also benefit greatly from porous sheathless CE-ESI-MS as demonstrated by a number of papers in profiling of biopharmaceuticals[5, 6]. Both the field of glycopeptide analysis and intact protein analysis are relatively new fields and suffer from the limitations in liquid chromatography technology, leaving the door open for CE-MS to make a significant impact.

Finally, porous sheathless CE-ESI-MS has shown to perform very well in our experiments but a number of potential developments could propel the technique to greater performance. The capillary length used in the experiments in **Chapters 6 and 7** was 90 cm's because this is the only length currently provided by the manufacturer. Optimal performance from these capillaries was obtained using ± 220 V/cm (20kV of separation system) resulting in EOF flow rates of ± 15 nl/min which is in the optimal range for the sprayer and provides a good separation window. Using separation capillaries that have lengths up to 135 cm will provide the same EOF flow rates and V/cm (30 kV over separation system) but will significantly increase the total peak capacity of the system, thereby improving the identification power when using the system for bottom-up proteomics. The use of neutral coating on the separation capillary like the approach that was taken in the experiments in **Chapters 1-3** could also potentially improve the performance of the currently available system. **Chapter 3** shows us the immense potential of the loadability and separation power of the porous sheathless CE-ESI-MS when a coating is applied to reduce the EOF in the system.

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Appendices

Summary

This thesis covers the progress that was made in the application of a porous sheathless interface in CE-ESI-MS analysis of protein digests. It has previously been shown that the porous sheathless interface could produce electrospray and separations at ultra-low flowrates and therefore had the potential to greatly improve the performance of CE-ESI-MS. Electrospray ionization at ultra-low flow rates has also shown to have significant advantages over conventional nano flow rates (±300 nl/min) not only regarding sensitivity but also minimizing ionization efficiency bias for specific compounds. The porous sheathless interface has shown to consistently generate a stable electrospray at flow rates below 10 nl/min and could therefore potentially produce the same ionization effects that were previously observed with regards to reduced ion suppression. **Chapter 1** investigates the potential of the porous sheathless interface at ultra-low flow rates in reduction of ion suppression in the ionization of phosphorylated peptides. For these experiments five synthetic peptides with a constant amino acid sequence and varying numbers of phosphorylations (0-4) were obtained. The observed effect was a significant increase in sensitivity for the multiply phosphorylated peptides with the greatest improvement (factor 4) for the peptide containing 4 phosphorylations. The ionization efficiency of the interface was also shown in the analysis of IgG 1 derived glycopeptides. Using a neutrally coated capillary the separation could be performed at flow rates below 10 nl/min and therefore large volume injection through transient isotachophoresis combined with ultra-low flow ionization resulted in a 20-fold improvement in sensitivity compared to the currently used platform (**Chapter 2**).

The separation at ultra-low flow rates is not only beneficial for the ionization process but equally important is the significant improvement in separation power that can be achieved using this approach. **Chapter 3** shows the potential of a period of Zero-Flow separation combined with large volume injection before eventual mass spectrometry. It was found that 100% increase in peak capacity could be achieved using this approach with minimal deterioration of peak shape therefore maintaining sensitivity. Such improvements in peak capacity can directly translate in quite large improvements in numbers of identified peptides in bottom-up proteomics of complex sample.

Although CE-MS does not belong to the mainstream methods used in bottom-up proteomics, a number of groups have explored CE as an alternative or complementary technique to the current standard nano-LC-MS. To obtain a perspective of the history and applications in the field of CE-MS proteomics and more specifically CE-ESI-MS bottom-up proteomics two in-depth literature reviews were performed. The first review (**Chapter 4**) looked at the field of CE-MS bottom-up proteomics as a whole and the

developments made in recent years. This review covered both CE-MALDI and CE-ESI-MS including the varying interfaces that are available for this technique. As the review in **Chapter 4** showed the promise of CE-ESI-MS for bottom-up proteomics a critical look was taken at all the required conditions for such a separation and analysis setup which resulted in the complete review presented in **Chapter 5**. In this review each aspect of the separation system is discussed separately and placed in the larger scope of developed applications to provide a novel user of CE-ESI-MS a frame of reference to start from. It was found that currently the best method of improving sensitivity is the use of large volume injections through a stacking approach which was therefore used throughout all experiments performed in this thesis.

Chapter 6 describes the development of a data processing workflow for the comparison of samples that are fractionated by SDS-PAGE. Because SDS-PAGE separates on basis of protein size/mass it is completely orthogonal to RPLC and CE separations making the fractions suitable to be analyzed by both techniques. A comparison of the two data sets would then show to what extent the two techniques are complementary in bottom-up up proteomics of more complex samples. The developed workflow showed excellent complementarity between the two strategies on both the identified peptide numbers and their hydrophobicity and peptide size. Moreover the data set from CE-MS identified more peptides and proteins.

The experiments in **Chapter 7** show the application of the sheathless CE-ESI-MS platform to the analysis of human glomeruli. A strategy similar to the one used in **Chapter 6** was applied to obtain in-depth proteomics knowledge on human glomeruli. Compared to the only previous in-depth experiment on human glomeruli, significantly more proteins were identified by our CE-MS platform. The analysis of laser micro dissected glomeruli from protocol needle biopsies is the ultimate goal in proteomics analysis of glomeruli. An investigation was performed by using isolated glomeruli from cadaver kidneys as a model sample to determine the number of proteins that can be identified from a certain amount of material. It was found that material equivalent to only one glomerulus was enough to identify more than 100 proteins using the developed strategy.

This thesis shows the promise of CE-ESI-MS using the porous sheathless interface for the analysis of post-translational modifications on proteins and for the analysis of protein digests. It was found that the low sample requirement and high sensitivity of CE-ESI-MS results in excellent sensitivity. Although, the sample loadability of the system is still a limitation, this is an engineering issue that should be solvable in the future. There are, therefore, strong indications that CE-ESI-MS with the porous sheathless interface can become a mainstream tool in the analytical environment.

Nederlandse samenvatting

Dit proefschrift beschrijft de vooruitgang die is gemaakt op het gebied van de koppeling van capillaire elektroforese aan massa spectrometrie door middel van een poreuze naald en het gebruik van deze koppeling in de analyse van gedigesteerde eiwitten. Er was al eerder aangetoond dat deze methode scheidingen bij hele lage vloeistofsnelheden toelaat en daarom de potentie had voor sterke verbeteringen in prestatie. Ook was aangetoond dat elektrospray bij hele lage vloeistof snelheden significante voordelen heeft in vergelijking met normale nano-flow snelheden (±300 nl/min), niet alleen wat betreft gevoeligheid maar ook wat betreft het verminderen van ion-suppressie.

De poreuze naald koppeling kan een stabiele spray genereren bij vloeistof snelheden lager dan 10 nl/min en zou daarom ook de eerder geobserveerde ionisatie effecten met betrekking tot verminderen van suppressie moeten kunnen bewerkstelligen. In **Hoofdstuk 1** is het potentieel van de poreuze naald koppeling bij hele lage vloeistofsnelheden voor de vermindering van ion-suppressie tijdens de ionisatie van gefosforileerde peptiden onderzocht. Voor deze experimenten zijn er 5 peptiden met nul tot vier fosforileringen gemaakt. Bij verlaging van de vloeistof snelheid van een infusie kon een significante verandering in de signaalintensiteit worden geobserveerd. De ionisatie efficiëntie van de koppeling werd ook aangetoond bij de analyse van glycopeptiden verkregen uit immunoglobuline G1. Er werd een neutraal gecoat capillair gebruikt waardoor de vloeistof snelheid onder de 10 nl/min kon worden gehouden. Hierdoor kon een groot monster volume worden geïnjecteerd en de gevoeligheid verbeterd met een factor 20 doormiddel van isotachoforese. (**Hoofdstuk 2**)

Lage vloeistof snelheden bij capillaire elektroforese verbeteren niet alleen het ionisatie proces maar ook kunnen significante verbeteringen in de scheiding met deze aanpak teweeg gebracht worden. **Hoofdstuk 3** laat het potentieel van de poreuze naald zien door de ontwikkeling van een methode waarbij een groot monster volume werd geïnjecteerd en vervolgens werd gescheiden bij een periode zonder vloeistof loop in het scheidingssysteem. Er werd aangetoond dat het gebruik van deze ‘nul vloeistof loop’ periode een 100% toename van de piek capaciteit teweeg bracht. Ondanks het feit dat CE-MS niet de meest gebruikte techniek is voor de analyse van eiwit-digesten zijn er een aantal groepen die onderzoek doen naar deze applicatie. Om een inzicht te krijgen van het gebruikt van CE-MS in het eiwit analyse veld zijn er twee overzichts artikelen geschreven. In **Hoofdstuk 4** is er naar het CE-MS eiwit digest analyse veld in het algemeen en de ontwikkelingen in de afgelopen jaren gekeken. Verschillende ionisatie-technieken en ontwikkelingen op het gebied van monster voorbewerking en scheiding strategieën werden besproken. Omdat **Hoofdstuk 4** vooral de potentie van

CE-ESI-MS voor de analyse van eiwit digesten toonde is er ook een nadere kritische blik geworpen op deze specifieke techniek. **Hoofdstuk 5** beschrijft de meest recente ontwikkelingen van CE-ESI-MS op het gebied van de analyse van eiwit-digesten. Het doel van dit hoofdstuk was het creëren van een basis voor mensen die onbekend zijn met deze techniek.

In **Hoofdstuk 6** is de ontwikkeling van een data verwerking strategie beschreven die gebruikt kan worden om twee monsters die gefractioneerd zijn met SDS-PAGE met elkaar te vergelijken. Omdat SDS-PAGE de eiwitten scheidt op basis van hun intacte massa is de methode complementair met de scheiding die wordt gedaan op de peptiden door middel van RPLC of CE. Hierdoor konden de SDS-PAGE fracties geanalyseerd worden met beide technieken. De ontwikkelde methode laat zien dat RPLC- en CE-scheidingen zeer complementair zijn voor wat betreft de identificatie van peptiden met betrekking tot hydrofobiciteit en peptide grote. Verder identificeerde de CE-MS methode in de zelfde fracties meer peptiden en eiwitten dan RPLC-MS analyse.

De experimenten in **Hoofdstuk 7** toonden de toepassing van de poreuze naald CE-ESI-MS methode bij de analyse van eiwitten die verkregen werden uit menselijke glomeruli. Een fractionerings methode die vergelijkbaar is met die beschreven in **Hoofdstuk 6** werd toegepast om zoveel mogelijk eiwitten te identificeren in een grote hoeveelheid glomeruli. Vergeleken met de enige eerder gepubliceerde resultaten de analyse van eiwitten uit glomeruli werd een significant groter aantal eiwitten geïdentificeerd. De analyse van glomeruli verkregen uit menselijke nier biopten is het uiteindelijke doel. Daarom werd onze CE-ESI-MS methode, gekoppeld met een geoptimaliseerde monster voorbewerking getest voor deze toepassing. Glomeruli verkregen door middel van laser micro-dissectie werden gebruikt als model monster. Er werd aangetoond dat er 100 eiwitten konden worden geïdentificeerd in een hoeveelheid materiaal dat equivalent is aan één volledige glomerulus.

Dit proefschrift toont de potentie van CE-ESI-MS waarbij gebruik wordt maakt van een poreuze naald voor de analyse van post-translationele modificaties op eiwitten en de analyse van eiwit-digesten. Er werd aangetoond dat deze strategie slechts lage monster hoeveelheden nodig heeft en zeer hoge scheiding resoluties kan behalen. Alhoewel de “laadbaarheid” van het systeem nog een beperkende factor is kan die in de toekomst verbeterd en/of opgelost worden door ontwikkelingen op het gebied van monster lading strategieën. Daarom is het mogelijk dat CE-ESI-MS met de poreuze naald een algemeen gebruikte analytische strategie kan worden.

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During my 4 years at the LUMC I only had the privilege to supervise one intern. Guinevere, thank you for your hard work and help during your period as a student and I'm very happy you found a spot as a PhD student in our group. Next up are naturally my two Para nymphs Frank and Hulda. I met Frank during my studies at the VU and though we did not interact to much then I was happy to hear he secured himself a position at the LUMC and has been a great friend and colleague ever since. Hulda, I met as part of 'team Iceland' during the 'fundamentals of analytical chemistry' course at the VU university. Shortly after she also started her masters internship at the LUMC and continued as a PhD student. You both have been a large part of making going to work fun as even when the science wasn't going so well the going to work was never dull.

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Curriculum Vitae

Anthonius (Anton) Adrianus Maria Heemskerk was born on October 3rd 1985 in Haarlem, the Netherlands to Johannes Nicolaas Maria Heemskerk and Anja Gijsbertha Maria Heemskerk - van der Aar. He attended the Rozenbeek grade school in Velserbroek, the Netherlands from grades 1 through 8. Subsequently, he attended the Mendel College schooling community and obtained his high school diploma at the Atheneum level in both the Nature & Science and Nature & Health profiles. His high school career was concluded with a year as a foreign exchange student at Cape Central High school in Cape Girardeau, Missouri, USA.

Anton's education was continued at the VU University in Amsterdam where he obtained a bachelor's degree in chemistry. During his bachelor's degree the major research project was performed under the supervision of Dr. Niels Martha in development of on-line reaction monitoring for a catalysed multi-component reaction and using mass spectrometric detection.

The bachelor internship sparked an interest in analytical sciences and resulted in the following of the 'analytical sciences' master program at the VU University. During this master program two internships were followed. A 3 month minor internship under the supervision of Dr. Gert van der Zwan and Dr. Michal Heger to research the influence of infra-red light on enzyme kinetics resulted in a publication in Journal Photochemistry and photobiology B. A 7-month master internship was supervised by Dr. John Stobaugh and Dr. Leon van Haandel at the University of Kansas, Lawrence, Kansas, USA. The internship covered the analysis of drug metabolites in human blood by HPLC-LIF and HPLC-MS and resulted in several publications.

Following the master's degree a PhD position in the development of CE-MS strategies for proteomics analysis became available at the LUMC. The results of research project can be found in this thesis. After the 4 year PhD period Anton continued his career as a CE application specialist with Sciex separations for which he moved to Germany in April 2015.

As of December 2015 he is back in the Netherlands in his current position as a lecturer/ Teacher in the field of (analytical) chemistry at the Hogeschool in Leiden.

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