Exploring the proteome by CE-ESI-MS

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Chapter 5

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

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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.

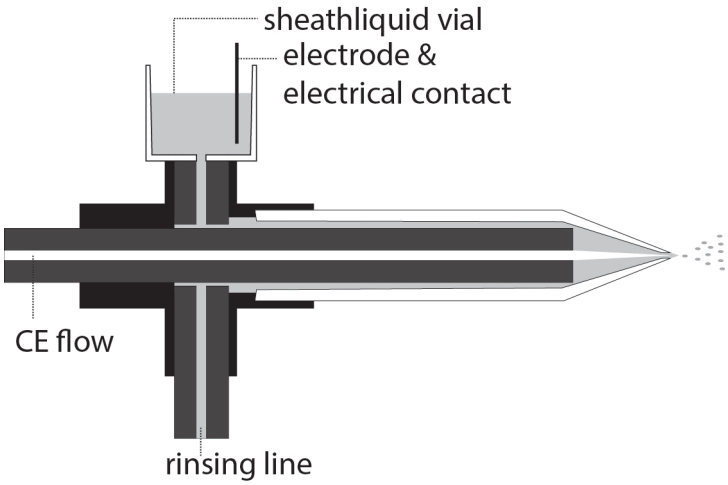

Figure 5-1: Schematic depiction of the electrokinetic junction nano sheathliquid interface as published by Dovichi and coworkers. [11]

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

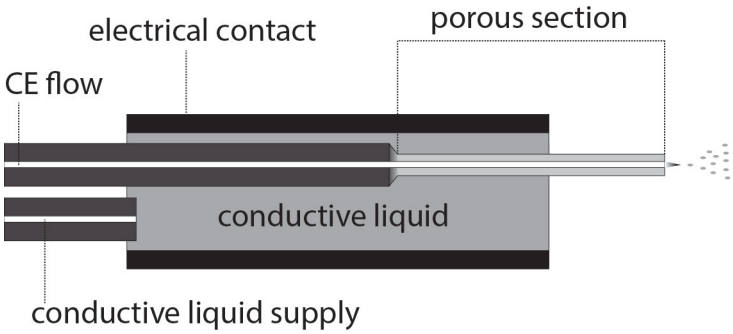

Figure 5-2: Schematic depiction of the porous sheathless interface as published by Mehdi Moini.[8]

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.

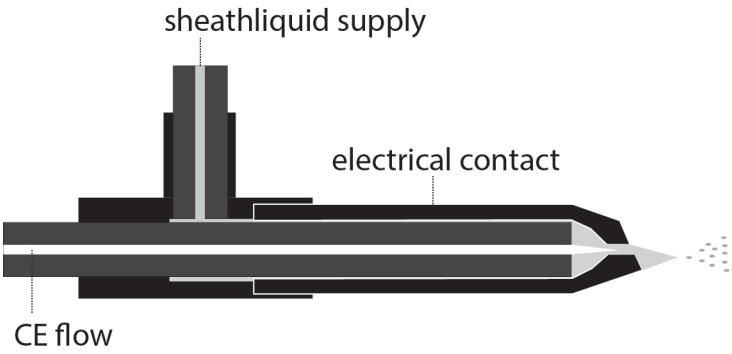

Figure 5-3: Schematic depiction of the junction at the tip interface as published by Chen and coworkers.[9]

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].

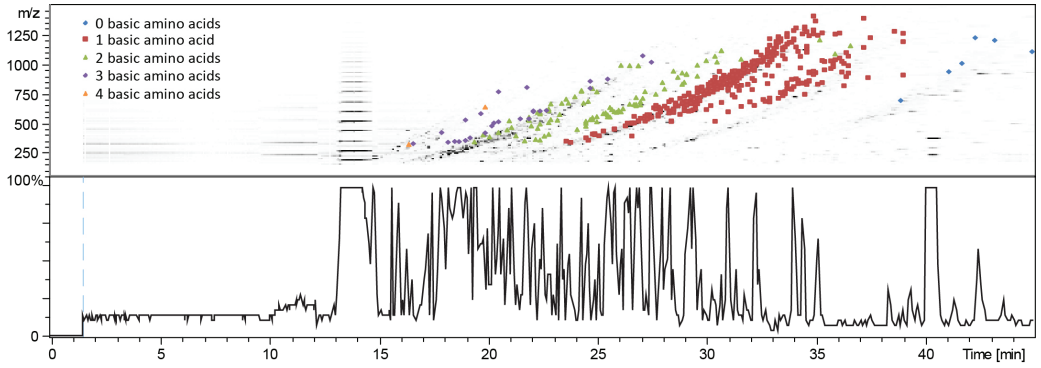

Figure 5-4: Separation of a complex cell lysate digest at pH 2.2. (A) shows the density plot of the observed masses in MS1 with the peptides that are identified and their number of basic amino acids. (B) Corresponding base peak electropherogram.

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

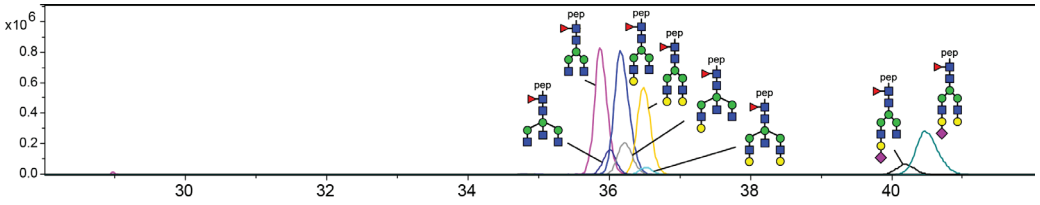

Figure 5-5: Separation of glycopeptides derived through IgG1 tryptic digest. (Figure adapted from [13])

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.

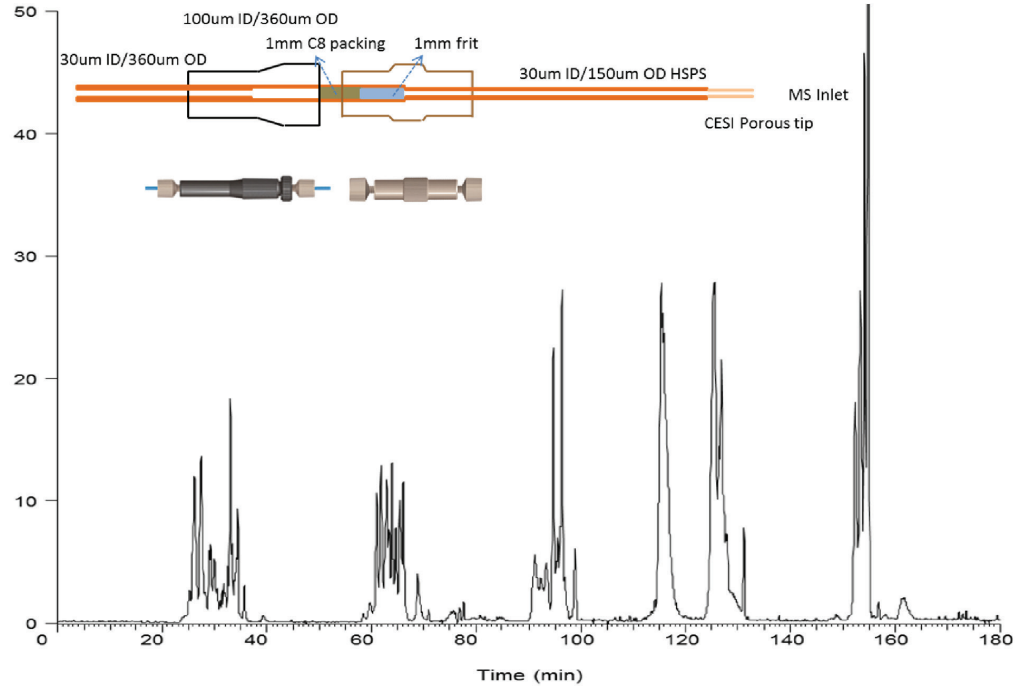

Figure 5-6: Schematic of the SPME-CE-MS/MS platform and base peak electropherogram for 5-step SPME multistep elution tITP-CE-MS/MS of 100 ng Pfu digest as published by Wang et al. [44]

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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