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Capillary zone electrophoresis for bottom-up analysis of complex proteomes

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Abstract

Capillary zone electrophoresis (CZE) is emerging as a useful tool in proteomic analysis. Interest arises from dramatic improvements in performance that result from improvements in the background electrolyte used for the separation, the incorporation of advanced sample injection methods, the development of robust and sensitive electrospray interfaces, and the coupling with Orbitrap mass spectrometers with high resolution and sensitivity. The combination of these technologies produces performance that is rapidly approaching the performance of UPLC-based methods for microgram samples and exceeds the performance of UPLC-based methods for mid- to low nanogram samples. These systems now produce over 10,000 peptide IDs in a single 100-minute analysis of the HeLa proteome.

1 Introduction

Bottom-up proteomics inevitably employs one or more stages of chromatographic separation of tryptic peptides before tandem mass spectrometry analysis. State-of-the-art analyses identify 4,000 proteins and nearly 35,000 peptides from the yeast proteome in a single 70 min analysis using 1.7- μ m diameter particles in reversed-phase liquid chromatography (RPLC) and an Orbitrap Fusion mass spectrometer [1], and over 5,000 proteins and 40,000 peptides from the HeLa proteome in a 90 min separation [2]. The performance of bottom-up proteomic analysis using chromatographic separation typically requires a microgram or more of sample; performance decreases dramatically with smaller loadings.

Capillary zone electrophoresis (CZE) is an alternative and complement to HPLC separation of tryptic peptides. Recent advances in column coatings, sample loading, background electrolyte, and interface design have resulted in dramatic improvements in the number of peptide and protein identifications in bottom-up proteomics. This review focuses on recent developments in the applications of CZE for bottom-up proteomics.

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Conflict of interest statement

Patents have been filed that cover the electrospray interface described in figures 2 and 3 and in references 18, 20, and 28. This technology has been licensed to CMP Scientific.

2 Capillary zone electrophoresis - basics

CZE is a remarkably simple separation method performed in a fused silica capillary filled with a background electrolyte [3]. A small plug of analyte is injected into the proximal tip of the capillary using pressure or by application of an electric field. The migration time of an ion through the capillary depends on its electrophoretic mobility ($\mu_{\text{electrophoresis}}$) along with the capillary length (L), the applied potential (V), and the electroosmotic mobility due to the charge on the capillary wall ($\mu_{\text{electroosmosis}}$)

$$time = \frac{L^2}{V (\mu_{\text{electrophoresis}} + \mu_{\text{electroosmosis}})} \quad (1)$$

The Debye-Huckel-Henry equation gives the electrophoretic mobility in terms of the ion's charge to size ratio

$$\mu_{\text{electrophoresis}} = \frac{z e}{6 \pi \eta r} \quad (2)$$

where z is the ion's charge, e is the charge on an electron, η is viscosity of the background electrolyte, and r is the ion's radius. The ion's radius is essentially invariant to experimental conditions, whereas charge can be manipulated by adjustment of the background electrolyte's pH.

Electroosmosis arises in a capillary with a charged wall. Under most experimental conditions, the fused-silica capillary wall takes on a negative charge. Cationic counter ions form a double layer at the wall, and the electric field drives these cations to the negatively charged electrode, typically at the detection end of the capillary. The movement of these ions drags solvent, generating bulk flow. Unlike pressure-driven flow, electroosmotic flow and electrophoretic migration have the important property of having a uniform profile across the capillary's cross-section, which eliminates band broadening due to the parabolic flow profile found in pressure-driven separations.

Separations in uncoated capillaries generate very strong electroosmotic flow, resulting in rapid separations. Electroosmosis can be manipulated through use of appropriate coatings on the wall of the separation capillary. Importantly, neutral coatings reduce electroosmosis, producing much slower separations.

Capillary electrophoresis can produce remarkably efficient separations. In a carefully designed system, longitudinal diffusion is the only source of band broadening, and theoretical plate counts of over 10^6 can be achieved [4]. However, in practice, such efficient separations are seldom observed. Instead, sample injection volumes contribute an extra-column source of band broadening, which degrades separation efficiency [4]. Joule heating produced by the electric field can generate a parabolic temperature profile across the capillary cross-section, which generates non-uniform flow profile due to the dramatic dependence of viscosity on temperature. Joule heating is important with large diameter capillaries, high ionic strength background electrolyte, and high electric fields. Another

source of peak broadening and peak distortion occurs when a high ionic strength sample is separated using a low ionic strength background electrolyte. In this case, the sample itself carries a significant fraction of the current, leading to distortion of the electric field along the length of the capillary. This source of band broadening arises when the ionic strength of the sample is greater than a few percent of the ionic strength of the background electrolyte.

3 CZE for bottom-up proteomics

CZE-MS has a long history as a tool for analysis of proteins. Richard Smith and colleagues were the first to interface CZE with mass spectrometry using electrospray ionization [5–7]. This early experiment used a single quadrupole mass analyzer and demonstrated separation of small amines, amino acids, nucleosides, and dipeptides using phosphate buffers for the separation and quite large capillaries (100- μ m ID, 1.0 to 1.5 m length), and generated separations with plate counts ranging from 7,000 to 600,000.

Early reports on the use of CZE coupled to a mass spectrometer for the analysis of tryptic peptides generated from a small number of standard proteins. In 1999, Tong *et al.* reported the first application of CZE for bottom-up analysis of a real protein sample, the yeast ribosomal proteins [8], and since then there have been a number of reports of the use of capillary electrophoresis for analysis of samples of increasing complexity [9–21]. One intriguing application of CZE has been for clinical analysis of the urinary and cerebral-spinal fluid peptidomes [22–24].

4 Performance of CZE for bottom-up proteomics applications

There has been a dramatic and rapid improvement in the performance of CZE for bottom-up proteomics over the past five years (Fig. 1), corresponding to a 3- to 10-fold increase in the number of peptide identifications per year. We begin this learning curve with Faserl's 2011 study, which identified 150 peptides generated by Arg-C digestion of histone proteins [10]. That study made the important observation that slower separations generate more peptide identifications. The authors used a neutrally-coated capillary and relatively high concentration (10% acetic acid) background electrolyte. The neutral coating reduces electroosmosis, and the acidic background electrolyte further reduces electroosmosis.

This group published its first application of capillary electrophoresis to bottom-up proteomics in 2012 [11]. We generated 11 fractions of the tryptic digest of the *M. marinum* secretome using reversed-phase RPLC, and analyzed those fractions using relatively rapid CZE separations; 334 peptides were identified in the study. Nano-RPLC analysis of the same digest yielded 388 peptide identifications in roughly the same analysis time. As an important observation, the CZE- and RPLC-analyses were complementary, and showed only modest overlap.

Also in 2012, Wang and colleagues coupled an on-column solid-phase microcolumn extractor with CZE separation for analysis of the *Pyrococcus furiosus* proteome; this was the first report of bottom-up proteomic analysis of a prokaryote using CZE [12]. They employed a 1-mm long reversed-phase microextractor at the proximal tip of the electrophoresis capillary, and performed five extractions using plugs of methanol-isopropanol mixtures and

a 95 mM acetic acid-5% MeOH background electrolyte with a poly-ethylenimine coated capillary. This coating produces a positive charge on the capillary wall, reversing electroosmosis. Over 2,300 peptides were identified from the digest using the five-step elution, and nearly 750 peptides were identified in a single-shot analysis. The authors compared CZE and RPLC for this analysis, and observed reasonable complementarity for peptide identifications. They also observed more peptide identifications for 100 ng samples using HPLC, and much better performance using CZE for mass-limited samples.

In 2013, we reported the identification of nearly 1,400 tryptic peptides from *E. coli* proteome in a single-shot CZE analysis [13]. That study compared HPLC and CZE for single-shot analysis of samples ranging from 1- to 100-ng. CZE significantly outperformed HPLC for mass limited samples. In the same year, we pre-fractionated the *E. coli* digest using off-line solid-phase extraction. Analysis of seven fractions generated nearly 5,000 peptides in a six-hour analysis [14].

In 2014, we reported the first application of CZE to the bottom-up proteomic analysis of a human cancer cell-line [19]. In that experiment, we used solid-phase extraction to capture the MCF-7 proteome; a fraction was eluted using acetonitrile and analyzed using CZE-MS/MS. Nearly 1,200 peptides were identified in that single shot analysis. Later that year, we collaborated with Josh Coon to couple CZE with an Orbitrap Fusion mass spectrometer [2]. This experiment used a neutral coated capillary and a 5% acetic acid background electrolyte. The study achieved over 10,000 peptide identifications from the tryptic digest of the HeLa proteome in a single-shot analysis. We also analyzed three fractions from the tryptic digest of the yeast proteome, which identified 15,443 peptides in five hours.

Two papers of note have appeared in the first half of 2015. One paper from this group describes an improved electrokinetically-pumped nanoelectrospray interface [20]. That system identified over 4,000 peptides in the single-shot analysis of the tryptic-digest of a *Xenopus laevis* egg using an Orbitrap Velos mass spectrometer. This analysis is noteworthy because of the very large amount of yolk protein present within the unfertilized egg, which dominates the spectral matches obtained in the analysis. Faserl and colleagues reported a comprehensive analysis of the yeast proteome using SILAC chemistry [21]. 182 fractions were generated from a large sample (1.4 mg) using reversed phase chromatography, and these fractions were separated using CZE in a neutral coated capillary with 10% acetic acid as background electrolyte. This study identified 33,656 tryptic peptides, which is the largest number of peptide identifications in a single proteomic analysis with CZE, albeit requiring over seven days of continuous instrument operation.

5. Sources of improvement

Figure 1 summarizes the improvements in CZE performance in terms of a learning curve, which is analogous to the famous Moore's law that has governed the performance of integrated circuits. While the performance of CZE is unlikely to continue an exponential improvement beyond the next year or two, it is instructive to consider the factors that have driven these advances, which can provide guidance for further improvements.

5.1 Improved electrospray interfaces for CZE

Starting with work in Richard Smith's laboratory [5–7], a number of interfaces between CZE and mass spectrometers have been developed [2, 25–28]. These advances have been described in a number of recent reviews [29–34]. The interface of CZE with a mass spectrometer faces several technical challenges. Among those challenges, electrical connection must be made to the distal tip of the separation capillary both to complete the electrophoretic circuit and to drive electrospray. This connection has been achieved through a number of approaches in both sheathless and sheath-flow designs. In both cases, care must be taken to avoid electrolysis and generation of bubbles, which disrupt the separation. The sheathless interface reported by Moini has been commercialized by Beckman (now Sciex) [27]. This design uses a capillary whose tip is etched to a few micrometer thickness. The thin-wall portion of the capillary can make electrical contact with a stagnant electrolyte, which is connected with the power supply. The elimination of sheath flow eliminates dilution of the sample. However, manufacture of the capillary is not trivial, and the fragility of the capillary can limit lifetime.

We have developed three variants of a sheath-flow nanospray interface for CZE [18, 20, 28]. In each case, the capillary is threaded through a plastic cross into a glass emitter (Fig. 2). The emitter is prepared using a commercial pipet puller. One side arm of the cross is connected by a transfer tube to a reservoir containing the sheath electrolyte, which is connected to a power supply to drive electrospray. By placing the high voltage connection at a distance from the emitter, we eliminate electrolysis within the emitter itself, reducing bubble formation that would disrupt the electrospray. The silicate interior of the glass emitter is negatively charged under most conditions, and generates electroosmotic flow. This flow is independent of the flow within the separation capillary, and pumps sheath flow around the separation capillary to form the electrospray. The electroosmotic flow is in the nanoliter/minute range, and the interface operates as a nanospray source.

We generated a Comsol model for the interface [28]. That model predicted, and experiment confirmed, that the signal intensity increases as the capillary tip approaches the exit orifice of the emitter. Our first-generation interface employed a flat tip at the end of the separation capillary (Fig. 3) [28]. The capillary butts against the interior of the conical emitter, limiting the distance from the capillary to the emitter to ~1-mm.

In a second-generation interface, we etched the distal end of the capillary tip to ~60- μ m diameter, which allowed the capillary to more closely approach the exit orifice, generating a ~10-fold improvement in signal [18]. This increase in signal is due, in part, to reduced diffusion and mixing of analyte as it traverses the distance from the capillary exit to the emitter orifice. We observed that the sensitivity improved with smaller emitter orifices, and our first- and second-generation interfaces used emitters with inner diameter of ~10- μ m. The etched tip is much thicker than that used in Moini's sheathless interface, and appears to be much more robust.

Unfortunately, the narrow emitter orifice tends to clog. Our third-generation interface combines an etched tip capillary with a relatively large emitter orifice ~35- μ m [20]. This wider emitter is much more robust than our earlier designs, and we routinely obtain many

days of continuous operation with the device. The wider emitter orifice also allows the capillary tip to more closely approach the orifice; the increased sensitivity from the reduced distance from the capillary exit to the emitter orifice counteracts the decreased sensitivity due to the larger orifice diameter, and the sensitivities of the second- and third-generation interfaces are similar. As one caveat, it is important that the sheath reservoir be at the same level as the emitter tip to minimize formation of a syphon, which would also contribute to the sheath flow.

5.2 Improvements in mass spectrometers

The improvement in mass spectrometer speed and resolution has driven a large portion of recent improvements in CZE performance, and improved mass spectrometer performance will undoubtedly lead to improvements in CZE performance. Electrophoretic peaks can be quite sharp; widths of a second or less are frequently observed. Very fast tandem mass spectrometers are required to generate spectra during CZE separation of peptides. While TOF instruments would seem to be ideal for CZE analysis, most recent work has employed Orbitrap instruments, where their high mass accuracy and reasonable speed have proven valuable.

5.3 Improved capillary coatings

Migration time is inversely related to the sum of electrophoretic and electroosmotic mobilities. Longer separations naturally lead to more tandem mass spectra and increased peptide identifications per run. As Faserl's 2011 study pointed out, the use of neutral coated capillaries is important to reduce electroosmosis to slow the separation [10]. Coated capillaries are commercially available from Polymicro and Beckman.

Although we have no experience with Beckman's capillaries, we have found that Polymicro's coated capillaries generate a significant variation in electrophoretic behavior. We recently described a simple and effective protocol that produces remarkably stable coating. This protocol relies on a reproducible initiation of the free-radical polymerization reaction that couples linear polyacrylamide to the capillary surface [35]. Figure 4 presents base peak electropherograms for the 1'st and 97'th injection of a BSA tryptic digest, corresponding to 145 hours of consecutive operation. Modest drifts in some components' migration time most likely are a result of evaporation of the running buffer over the six days of continuous operation. Note that successive injections are performed without flushing or rinsing the capillary in this experiment.

5.4 Large volume injections

In classical CZE, sample volumes of a few nanoliters or less are injected for analysis. These minute sample loadings limit the amount of sample taken for analysis, severely stressing the mass spectrometer's sensitivity for deep proteomic analysis. Improvements in sample loading are based on a number of simple protocols. In stacking, the sample is resuspended in a low conductivity electrolyte. A relatively large volume is injected onto the capillary using pressure. A large fraction of the applied voltage is dropped across this low conductivity region, leading to sample concentration at the injection end of the capillary. We used this stacking condition in the HeLa experiment, which allowed injection of ~400 ng of digest.

We have used the pH junction for large volume injections in CZE [36]. In the pH junction, peptides are prepared in a basic buffer and injected using pressure into the separation capillary. The background electrolyte is acidic, typically 10% acetic acid. Upon application of an electric field, the negatively charged peptides within the basic sample plug migrate toward the proximal tip of the capillary, where they are neutralized by the acidic background electrolyte, forming a focused plug. Once the basic sample buffer is neutralized, the peptides take a positive charge and migrate to the capillary's distal tip, where they are sprayed into the mass spectrometer. Quite large sample volumes, up to 25% of the capillary volume, are used for injection without excessive band broadening.

Yates and colleagues introduced the concept of preconcentration on a solid-phase material immobilized at the proximal tip of the capillary, followed by elution using a series of buffers [8]. In this protocol, a reversed-phase solid-phase microextraction column was used for preconcentration. Relatively large sample volumes were captured on the extractor and eluted using an alcohol-based elution system. A series of elution buffers of increasing strength can be used to fractionate the sample before CZE analysis. This system can be automated and provides a facile approach to sample preconcentration, fractionation, and CZE analysis while minimizing manual sample manipulations.

We reported a similar system that employs a strong cation exchange monolith for sample concentration [37]. In this case, peptides are prepared in an acidic buffer and loaded onto the monolith. A basic buffer is used to elute peptides. The eluted peptides are in a basic environment and form a pH junction in the acidic background electrolyte. The combination of solid-phase preconcentration and pH junction leads to remarkable levels of preconcentration. Sample volumes an order of magnitude large than the capillary volume can be loaded onto the capillary with little degradation of separation efficiency.

Elution buffers of increasing pH can be coupled with the on-line strong cation exchange monolithic preconcentrator to generate fractions for CZE analysis [38]. There is remarkably little overlap in peptide identifications between successive pH bumps, and the system provides a powerful tool to analyze complex proteomes.

6 CZE for high sensitivity proteomics

CZE has been compared with HPLC for the bottom-up analysis of a number of proteomes [10–13, 15]. Inevitably, HPLC outperforms CZE in terms of the number of peptide and protein identifications for injection amounts greater than ~100 ng, and CZE outperforms HPLC for samples smaller than ~10 ng. The improved performance of CZE likely reflects higher peak intensity in CZE due to higher efficiency separations. In addition, CZE provides a separation that does not rely on partitioning on a stationary phase, dramatically decreasing the probability of irreversible peptide loss on active sites.

To demonstrate the sensitivity of our nanoelectrospray interface, we analyzed 16 pg and 400 fg of *E. coli* digest (Fig 5) using a 40-cm long, 10- μ m inner diameter separation capillary coupled to an Orbitrap Q-Exactive mass spectrometer with our second-generation interface [18]. Extracted ion electropherograms were generated (with 3 ppm mass accuracy) for 154 peptides and summed for the figure; no smoothing was applied. The most abundant peptide

in the *E. coli* proteome is from elongation factor Tu, which was present at ~100 zmol in the 400 fg electropherogram. This peptide generated a signal-to-noise ratio of ~300, and it is expected that the peptide would be present at ~1 zmol level at the detection limit (3 s). Of course, lower abundance peptides will likely suffer from ion suppression and are expected to have poorer detection limits. Nevertheless, these data demonstrate the extremely efficient transfer of ions from the separation capillary to the mass analyzer in this experiment.

We point out one limitation of the experiment used to generate Figure 5. The volume of the entire capillary used in this experiment is only 30 nL, and only ~40 pL of a 0.01 mg/mL digest was injected onto the capillary. While only fg amounts of digest were injected, a much larger sample is required for the analysis due to challenges in manipulating pL volumes.

7 What next?

Although CZE has had a dramatic improvement in performance over the past few years, it still lags behind RPLC in terms of single-shot performance. We reported state-of-the-art single-shot proteomic analysis for the HeLa proteome using both CZE and HPLC with the same Orbitrap Fusion mass spectrometer [2]. CZE identified ~10,000 peptides across this separation whereas HPLC identified over 40,000 peptides. Perhaps the most instructive way to compare the experiments is to look at the protein identification rates across the separations (Fig 6). RPLC generates a uniform peptide identification rate of over 10 peptides per second across a large fraction of the 80 minute separation window. In contrast, CZE generates roughly half the identification rate across a window that is roughly half the duration of the RPLC separation.

We used an unsupervised least-squares routine to fit a Gaussian function to the most intense parent ion peaks. The median peak width, measured as the standard deviation of the Gaussian function, was 8.5 s, compared to a median peak width of ~3 s for the HPLC data. The relatively broad peak observed in the CZE data reflects the injection conditions, which relied only on stacking to concentrate the sample. Optimized injection conditions will be required to increase the peptide identification rate to equal that of HPLC.

Further improvements in the number of peptide identifications will come from optimization of the separation conditions. Figure 7 presents the peptide *m/z*, along with the peptide identification rate from figure 6. There is a modest correlation between migration time and peptide *m/z*, with higher *m/z* value peptides migrating later. This correlation arises from the inverse relationship between mobility and peptide size in equation 2. The dense band extending from 20 minutes and 400 *m/z* to 70 min and 1200 *m/z* contains the vast majority of peptides observed in the experiment. It is clear that improvements in peptide identifications will arise from separation conditions that spread these peptides over a larger migration time window.

There are several strategies to modify the separation window. First, the electric field can be adjusted during the run to slow the separation during the period of densest peptide migration, providing more opportunity for generation of tandem spectra [39]. Second, the separation pH can be adjusted to higher values. The acetic acid background electrolyte used

in this experiment essentially protonates all basic groups; the peptide's charge is determined by the number of unmodified arginine, histidine, and lysine residues. Operation at slightly more basic conditions will lead to partial deprotonation of aspartic and glutamic acid residues, generating partial negative charge, slowing and spreading the separation window. Third, and most simply, the capillary length can be increased, leading to a slower separation.

The dead time at the start of the electropherogram reflects the time necessary for the fastest component to migrate through the capillary. This period appears to represent wasted time wherein analyte is not present within the mass spectrometer. In some cases, however, this time can be recovered by injecting the next sample before the previous sample has completed its separation. For example, the twenty-minute dead time can be recovered by injecting a second sample 20 minutes before the end of the preceding electrophoretic run.

The number of peptide identifications generated by CZE is within a factor of ~4 of the state-of-the-art HPLC single shot analysis of complex proteomes. However, a simple comparison of IDs per run is slightly misleading. HPLC requires time both for sample loading and column regeneration between successive runs. In contrast, CZE requires no column rinsing or regeneration between runs, and sample loading is quick and simple. Furthermore, CZE instrumentation is simple and inexpensive, particularly if the capillary is coated in-house.

Finally, this review neglects discussion of the use of CZE for quantitative analysis, analysis of post-translational modifications, for top-down proteomics, and for glycan analysis. Given support from funding agencies, it is quite likely that CZE will expand to the study of additional research topics. We conclude this review by mentioning an exciting application of CZE for metabolomics analysis. Onjiko and colleagues have recently published the application of CZE for analysis of single blastomeres isolated from early-stage *Xenopus laevis* embryos [40]. While single cell proteomics has been performed on *Xenopus* embryos [41], the generation of single blastomere metabolomics analysis provides opportunities for characterizing fate-determinants during early vertebrate development.

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Figure 1. Learning curve for bottom-up proteomic analysis using CZE-MS since 2011. Reference numbers shown above each datum.

Figure 2.

Electrokinetically-pumped sheath-flow nanoelectrospray interface. Electroosmosis at the borosilicate emitter pumps the sheath flow at nanoliters per minute [28].

Figure 3.

Three generations of the electrokinetically-pumped sheath-flow nanospray interface. The first-generation interface uses a flat tipped separation capillary and a ~10-μm exit orifice [28]. The second-generation interface uses an etched tip separation capillary, which allows the tip of the capillary more closely approach the exit orifice [18]. The third-generation interface combines the etched tip capillary with a much larger exit orifice, dramatically reducing plugging of the emitter [20].

Figure 4. Butterfly plot of the base peak electropherogram of a BSA digest using a thermally-initiated polymerization protocol (data from [35]). The top plot is of the first electropherogram and the bottom plot is the 97th electropherogram, generated after ~145 hours of continual operation.

Figure 5.

Butterfly plots of extracted ion electropherogram of *E. coli* digests, presented as butterfly plots. A. Duplicate analyses of 16 pg of digest injected onto the capillary. B. Comparison of extracted ion electropherograms of 16 pg (top) and 400 fg (bottom). The 400 fg signal was multiplied by 40 to scale by the injected amount. Data from ref [18].

Figure 6. Peptide identification rate for CZE and RPLC analysis of the same HeLa proteome digest using the same Orbitrap Fusion mass spectrometer. Data from ref [2].

Figure 7. Details of the CZE analysis of the tryptic digest of the HeLa proteome. Top – peptide m/z values vs migration time. Bottom – peptide identification rate. Data from ref [2].