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Capillary zone electrophoresis as a tool for bottom-up protein analysis

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Background

A small community of researchers is reinvestigating capillary zone electrophoresis-mass spectrometry (CZE-MS) for protein analysis. CZE provides a remarkably simple separation mechanism, very low cost instrumentation, and very high-resolution separations. Depending on its operational conditions, CZE can provide rapid separations for high-throughput analysis, or it can provide slow separations for deep bottom-up analysis of complex proteomes.

This effort has produced a remarkable improvement in performance. One metric is the number of detected peptides in a bottom-up single-shot analysis of complex proteomes, which has averaged roughly a 10-fold improvement in each of the last three years. While pharmaceutical and clinical researchers may not require analysis of complex proteomes, the experience gained in proteomic analysis provides guidance for analysis of less complex samples.

This commentary highlights several challenges that have been faced and solutions demonstrated in CZE analysis of proteins.

Challenge 1 – robust electrospray interface

CZE-electrospray interfaces present a serious design challenge. The interface must provide electrical contact at the distal end of the separation capillary in order to both complete the electrophoretic circuit and provide potential to drive the electrospray.

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The first CZE electrospray interfaces were developed in the 1980s and were essentially modified HPLC interfaces [1]. These devices pump a sheath fluid at μL/min rates over the distal tip of the separation capillary and through a metal emitter, often with nebulizing gas to help stabilize the spray. The metal tip provides a convenient means of applying potential to the interface. The high sheath flow rate used in these early interfaces results in significant sample dilution.

Two interfaces were developed in the 2000s. In a sheathless interface, the distal tip of the separation capillary is etched to a few micrometer thickness [2]. The thin section is immersed in an electrolyte solution held at the electrospray potential, and the thin capillary wall provides sufficient conductivity to support electrospray. The absence of a sheath fluid eliminates dilution of the sample in the interface but requires that the background CZE electrolyte must support electrospray, which typically requires use of acidic solutions with volatile (*e.g.* methanol or acetonitrile) additives.

We have reported three generations of a nanospray interface based on electrokinetic pumping of a sheath liquid [3–5]. In these interfaces, a glass emitter is drawn to a 5- to 40-μm diameter tip using a micropipette puller. The separation capillary is threaded into this emitter and held in place using a plastic ‘T’ or cross. The sidearm of the T is connected to a sheath reservoir, and potential is applied to that reservoir to drive electrospray. Electroosmosis at the interior surface of the glass emitter generates very stable flow of the sheath fluid in the nL/min range. The use of acidic sheath electrolytes with 10–50% (v/v) organic additive produces very stable spray without the need to add organics to the CZE background electrolyte. The latest generation of the interface is quite robust, and continuous operation over many days is routinely achieved.

Challenge 2 – limited sample loading

To achieve the highest efficiency separation, CZE requires sample loadings of about 1 nL [6]. This minute sample volume can produce separations that approach a million theoretical plates. Unfortunately, this small sample volume contains a miniscule amount of analyte, severely stressing detection sensitivity.

Fortunately, several sample concentration methods have been developed that allow injection of very large sample volumes without serious degradation of separation efficiency. We highlight three: stacking, pH Junction, and online Solid Phase Extraction (SPE).

In stacking, the sample is prepared in a low ionic strength electrolyte. The low ionic strength sample has correspondingly low conductivity that produces a high electric field across the sample plug, which concentrates the sample. A well-desalted tryptic digest will have ionic strength that is an order of magnitude lower than the background electrolyte, allowing injection of tens of nanoliters of sample without excessive band broadening. We have shown that stacking can be used to load ~400 ng of tryptic digest to identify over 10,000 peptides and 2,100 proteins from the single-shot analysis of a HeLa tryptic digest in 110 min [7].

In the pH junction [8], tryptic peptides are prepared in a basic solution where the amphiprotic peptides are negatively charged. The sample is then pressure-injected into a

capillary filled with an acidic background electrolyte. Upon application of an electric field, the negatively charged peptides migrate toward the positive, injection end of the capillary. Analyte are neutralized and concentrated when they reach the acidic background electrolyte. Concentration continues until the re-suspension buffer is depleted, at which point the peptides acquire a positive charge and undergo conventional migration and separation. This process requires no manipulation of the electrophoresis beyond preparation of the sample in a basic electrolyte. The pH junction can be used to inject hundreds of nanoliter samples, corresponding to over 30% of the capillary volume, with minimal loss of separation resolution [9].

Finally, a plug of stationary phase can be immobilized at the proximal tip of the separation capillary for SPE from very large volumes of sample. We typically employ a strong cation exchange solid-phase monolith for SPE and an acidic background electrolyte [10, 11]. Tryptic digests are prepared in an acidic solution, where they carry a positive charge and are retained on the monolith. Components are eluted with a plug of a low ionic strength basic solution. Since the elution solution has lower ionic strength than the background electrolyte, stacking is produced upon application of an electric field. More importantly, the basic elution solution forms a pH junction, leading to additional band sharpening.

Challenge 3 – control of separation speed

CZE is known for providing fast separations, which are valuable in high-throughput screens and other applications [12]. However, slow separations are desirable when performing deep proteomic analysis of complex samples; slower separations allow time to generate more tandem spectra for identification of large number of components. Separation speed can be controlled by adjusting the separation voltage, capillary length, and extent of electro-osmosis. We find that the use of capillaries coated with a neutral polymer, such as linear polyacrylamide, coupled with a 1 M acetic acid background electrolyte provides very low electro-osmosis and advantageous separation conditions [7]. While there are commercially available coated capillaries, those capillaries tend to be expensive and can suffer from poor consistency. We have published a simple protocol for the preparation of the coated capillaries that results in a reproducible product [13].

CZE *vs* UPLC

Ultraperformance liquid chromatography (UPLC) has had many years of development, and is currently the separation mechanism of choice in protein and peptide analysis. CZE was first applied to a whole organism proteomic analysis two years ago, and is at a nascent stage of development. Nevertheless, there have been dramatic improvements in the performance in CZE in the past two years, and those improvements will undoubtedly continue as more experience is gained.

At this point in time (autumn 2015), we can make several observations. The loading capacity of CZE is roughly an order of magnitude lower than typical UPLC systems, and UPLC inevitably produces more peptide and protein identifications when large sample amounts are available. The state-of-the-art in the number of peptide and protein identifications produced in a single run is about three times larger for UPLC than CZE [7].

CZE and UPLC produce similar numbers of protein and peptide identifications for ~50 ng samples, and CZE provides superior performance for smaller sample loadings [14–16]. It is clear that improved sample loading capacity will improve the performance of CZE, and the performance difference between CZE and UPLC will likely continue to shrink.

However, most protein analysis problems facing the clinical and pharmaceutical industries do not require a large numbers of protein and peptide identifications from a whole proteome. Instead, quality control for recombinant therapeutics and identification of biomarkers dominate routine analyses. In those cases, the reproducibility, resolution, and simplicity of CZE become very attractive.

We conclude by mentioning three other applications of CZE. First, the technique has been applied to the top-down analysis of intact proteins [17–22]. Analysis of these proteins is challenging because of the requirement of a sophisticated mass spectrometer, complex data analysis, and the presence of a range of proteoforms for each protein; these proteoforms are decorated with different sets of post-translational modifications that confound bottom-up analysis. Top-down analysis is ideal for characterizing the various modifications present on a protein because the proteoforms are not comingled as in bottom-up approaches. Typical applications are in the characterization of recombinant antibodies used in the biopharmaceutical industry. Second, CZE has proved to be a very powerful tool in the characterization of glycans [23–25]. Third, CZE is finding application in the clinical analysis of cerebral spinal fluids and other samples [26–27]. These and other applications of CZE will benefit from further improvements in sample loading, electrospray interface performance, and control of separation speed.

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