Published in final edited form as:

Curr Opin Chem Biol. 2013 October ; 17(5): . doi:10.1016/j.cbpa.2013.07.018.

High sensitivity capillary zone electrophoresis-electrospray ionization-tandem mass spectrometry for the rapid analysis of complex proteomes

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Abstract

The vast majority of bottom-up proteomic studies employ reversed-phase separation of tryptic digests coupled with electrospray ionization tandem mass spectrometry. These studies are remarkably successful for the analysis of samples containing micrograms of protein. However, liquid chromatography tends to perform poorly for samples containing nanogram amounts of protein, presumably due to loss of trace-level peptides within the chromatographic system. Capillary zone electrophoresis provides a much simpler flow system and would appear to be an attractive alternative to liquid chromatography for separation of small peptide samples before electrospray ionization and mass spectrometry detection. However, capillary zone electrophoresis has received very little attention as a tool for analysis of complex proteomes. In 2012, we reported the use of capillary zone electrophoresis for the analysis of the secretome of *M. marinum*, a model system for tuberculosis. Roughly 400 peptides and over 100 proteins were identified from this medium-complexity proteome; this identification required analysis of a set of 11 fractions and occupied three hours of mass spectrometer time. We have recently employed an improved capillary zone electrophoresis system for the analysis of 100 ng of the *E. coli* proteome and observed over 1,300 peptides and nearly 350 proteins in a single separation. More interestingly, analysis of 1 ng of the *E. coli* proteome yielded over 600 peptide and 140 protein groups. This sample size approaches that of a large eukaryotic cell, suggesting that capillary zone electrophoresis may ultimately be a useful tool for chemical cytometry.

Introduction – single cell proteomics

A eukaryotic cell is typically 10- to 20-micrometers in diameter, has a volume of 0.5 to 5 pL, and has a mass of 0.5 to 4 ng. If the cell is 30% protein by weight, then the cell contains from 0.15 to 1.2 ng of protein; a value of 0.5 ng/cell is often quoted [1]. Analysis of this minute amount of protein is a formidable challenge. Selected proteins can be identified by immunostaining and detected with high sensitivity by flow or image cytometry [2–7]. While able to characterize a very large number of cells, classical cytometry methods are not able to characterize large numbers of components within cells.

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Chemical cytometry employs modern analytical methods to characterize the composition of single cells [8–12]. These analytical methods are usually destructive, wherein the cell is lysed and its components analyzed. For example, Arriaga and colleagues have employed capillary zone electrophoresis and laser-induced fluorescence to characterize the mitochondrial population within single cells [13–17].

We have used fluorogenic labeling and Capillary zone electrophoresis to characterize the composition of single cells [18–21]. This technology provides exquisite sensitivity, typically 100 yoctomoles for many proteins. Two-dimensional capillary electrophoresis analysis of single cells also produces several-hundred resolution elements [20–21]. However, fluorescence is inherently an information-poor technology, and identification of components represents a heroic effort [22].

High sensitivity proteomics by mass spectrometry

Mass spectrometry is an extraordinarily information-rich technology that is capable of detecting tens of thousands of peptides generated in a single separation. Identification of large numbers of peptides inevitably is obtained from samples consisting of micrograms of proteins. Analysis of samples containing less than 100 ng protein is challenging using conventional mass-spectrometry based analysis methods [23–24].

The vast majority of proteomic studies employ a bottom-up strategy, where the cellular lysate is digested with trypsin to create a pool of peptides. These tryptic peptides are usually fractionated by strong cation exchange chromatography, and the fractions are then analyzed by reversed-phase liquid chromatography that is coupled to a tandem mass spectrometer through electrospray ionization. There are a relatively large number of manual manipulations involved in sample preparation and fractionation, which lead to sample loss. Mann's group has developed technologies that minimize sample loss, and has used those technologies to analyze the proteome of a single kidney glomeruli and a single pancreatic islet of Langerhans [25]. In these analyses, proteins are extracted from the tissues, digested with trypsin, and separated in an analytical column. These micro-organs contained a few thousand cells and the protocol identified nearly 7,000 proteins using tandem mass spectrometry.

The few examples of single-cell protein analysis by mass spectrometry focus on detection of hemoglobin in single erythrocytes, where that protein accounts for ~90% of the erythrocyte's protein content, either using top-down analysis of intact hemoglobin molecules in a single-cell lysate by electrospray ionization and Fourier transform ion cyclotron resonance mass spectrometry or MALDI-TOF mass spectrometry [26–27]. Imaging MALDI mass spectrometry has also proven valuable for the analysis of neuropeptides found in single neurons [28–30], although comprehensive analysis of proteins from single cells remains a formidable challenge.

Capillary zone electrophoresis

Development tools for bottom-up proteomic analysis of single cells will be challenging. This task will require the efficient digestion of proteins and the efficient separation and identification of the resulting peptides. Conventional chromatographic approaches employ relatively complex fluidics, which provide opportunities for loss of peptides [31–33].

Capillary zone electrophoresis provides an intriguing alternative to liquid chromatography for trace-level proteomics [34–37]. Capillary zone electrophoresis is performed in a fused silica capillary that provides very little surface area for sample loss. The narrow diameter capillary, typically 50- μm , is ideally suited for manipulation of small volume samples. The

separation efficiency provided by Capillary zone electrophoresis routinely exceeds 100,000 plates, so that the separated components tend to be at much higher concentration than produced by liquid chromatography.

There have been at least two challenges in applying Capillary zone electrophoresis for high sensitivity analysis of complex proteomes. First, the efficient peaks produced by Capillary zone electrophoresis require very fast mass spectrometry to generate useful tandem mass spectra during the fleeting duration of the electrophoretic peak. The Orbitrap series of mass spectrometers has proven to be very useful detectors in Capillary zone electrophoresis. Second, commercial electrospray interfaces suffer from significant limitations [38]. Sheathless interfaces tend to be fragile and complicated, and require that the separation buffer also supports electrospray [39]. Sheath-flow interfaces tend to subject the sample to unacceptable dilution. We have developed and applied an electrokinetically pumped sheath-flow electrospray interface [40–48]. This interface operates in the nanospray regime, which minimizes dilution but allows introduction of electrospray-friendly solvents after the separation. The interface produces stable spray and has been applied for both capillary zone electrophoresis and capillary isoelectric focusing analysis of standards and complex proteomes.

There have been a handful of examples of Capillary zone electrophoresis analysis of real-world protein samples. Faserl *et al.* evaluated a porous-tip electrospray interface for CE-ESI-MS/MS analysis of Arg-C-digested rat testis linker histones, identifying 135 peptides and eight non-histone H1 proteins from 6 ng of sample [49]. Wang *et al.* developed on-line solid phase microextraction preconcentration, fractionation, and transient isotachopheresis CE–ESI-MS/MS procedure with the porous tip interface for proteomic analysis for a series of sample loading amounts, and identified 584 peptides from duplicate analysis of 5 ng of *Pyrococcus furiosus* tryptic digest [50].

Our group has used the electrokinetically-pumped electrospray interface for the bottom-up analysis of the tryptic digest of a sample of intermediate protein complexity, the secreted protein fraction of *Mycobacterium marinum* [41]. Eleven fractions were generated by reversed-phase LC (RPLC) and each fraction was analyzed by the CZE-ESI-MS/MS system. In total, 334 peptides corresponding to 140 proteins were identified in 165 min of mass spectrometer time from ~1 ng of sample. Roughly 40 peptides were detected in each 15-min long capillary zone electrophoresis separation.

Figure 1 presents a base peak electropherogram from analysis of a single reversed-phase liquid chromatographic fraction prepared from *M. marinum*. The electropherogram is quite sparse, consisting of half a dozen peaks of reasonable intensity. A total of 54 unique peptides were identified from this data. Clearly, this quality electropherogram, which was state-of-the-art for 2012, is of unacceptable quality for characterizing many components within minute samples.

Improved Capillary zone electrophoresis proteomics

Until very recently, the largest number of identifications in a single CE analysis (single-shot analysis) is on the order of 50 peptides [39,41]. This disappointing performance might be due to several reasons. Most importantly, only relatively simple samples have been analyzed, which obviously limits the number of peptide and protein identifications. Also, the relatively fast separations produced by Capillary zone electrophoresis limit the number of tandem mass spectra that can be accumulated during a run. In addition, detection of low abundance components is limited by the nanoliter volume loading capacity of capillary zone electrophoresis.

We addressed these issues in a recent publication that reported the use of capillary zone electrophoresis coupled to the electrokinetically-pumped sheath flow electrospray interface for the analysis of the tryptic digest of the *E. coli* proteome [47]. This sample was of significantly higher complexity than that used in earlier studies. This separation used a coated separation capillary, which resulted in a slower separation that produced the generation of more tandem spectra than our earlier work. Finally, the sample was prepared in a low conductivity buffer, which allowed injection of a larger volume sample under stacking conditions, which resulted in very efficient peptide separation.

Figure 2 presents three electropherograms for the separation of 100, 10, and 1 ng of *E. coli* proteome digest. The complexity of the electropherogram is significantly increased compared to figure 1.

Triplicate analyses were performed. $1,377 \pm 128$ ($\pm$ standard deviation of the distribution) peptides were identified from each 100 ng injection. The number of peptide identifications decreased logarithmically with sample amount, Figure 3. Triplicate analysis of a 10 ng sample identified 997 ± 54 peptides. Triplicate analysis of a 1 ng sample produced 627 ± 38 peptide IDs and 879 ± 41 peptide spectral matches. The 1 ng results were obtained from 2000 ± 200 tandem spectra, corresponding to a detection efficiency of over 40%.

Separation efficiency is remarkably high and migration times are remarkably reproducible. As an example, Figure 4 presents triplicate selected ion electropherograms generated at m/z 693.39 (VIELQGIAGTSAAR, a peptide from D-ribose-binding periplasmic protein) for 1-, 10-, and 100 ng digests. A nonlinear least squares algorithm was used to fit a Gaussian function to the peaks. Triplicate analyses of the 1-ng sample resulted in separation efficiencies of $105,000 \pm 35,000$ plates. The relative standard deviation in migration time was 1.6%, and the relative standard deviation in peak height was 6%. Migration time and separation efficiency are consistent for the 1- and 10-ng samples. However, there was a mobility shift and degradation in separation efficiency for the 100-ng sample. The mobility shift is due to slightly lower voltage applied (250 V/cm) for 100 ng compared with that from 1ng and 10ng samples (300 V/cm). The degradation of separation efficiency was likely due to the high ionic strength produced by the peptides within the sample, which both decreased stacking and distorted the electric field during the separation, leading to a loss of separation efficiency.

Prospects for single-cell bottom-up proteomics

The results produced by analysis of a 1 ng tryptic digest are interesting. This amount of sample corresponds to the protein content of a single $\sim 20\text{-}\mu\text{m}$ diameter cell, and suggests that Capillary zone electrophoresis is capable of resolving over 600 peptides from such a sample. However, several formidable hurdles remain before single cell proteomics using mass spectrometry can tackle these samples.

To perform bottom-up proteomics on a single eukaryotic cell, it will be necessary to lyse the cell and digest proteins before separation and mass spectrometry analysis. This group and others have developed protocols for introducing a single cell into a capillary and lysis through osmotic shock or the action of surfactants [18]. We have also developed an on-column proteolytic digester based on immobilized trypsin [46, 51]. This device provides efficient digestion of minute amounts of protein. Combination of single cell aspiration and lysis, on-column digestion, and capillary zone electrophoresis separation should provide access to a reasonably large number of tryptic peptides from a single cell. Further advances will come from use of multiple-reaction monitoring and accurate mass and time tags [44], which improves detection limits by roughly an order of magnitude for selected peptides.

Acknowledgments

We thank Dr. William Boggess in the Notre Dame Mass Spectrometry and Proteomics Facility for his help with this project. This project was supported by a grant from the National Institutes of Health (R01GM096767).

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•• Of outstanding interest

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Highlights

- We employ capillary zone electrophoresis for bottom-up proteomics.
- Nanogram amounts of the tryptic digest of the *E. coli* proteome are analyzed.
- > 600 peptides are identified in a single run of a 1 ng sample.
- This sample amount approaches the protein content of a single eukaryotic cell.

Figure 1. CE-ESI-MS/MS analysis of an HPLC fraction of the tryptic digest of the *M. marinum* secretome, the state of the art in 2012.

Figure 2.
Separation of 100, 10, and 1 ng of an *E. coli* tryptic digest by capillary zone electrophoresis.
The number of peptide identifications is noted in each panel.

Figure 3.

Average number of peptide IDs versus the amount of sample loaded for single-shot Capillary zone electrophoresis analysis of the *E. coli* proteome. Line – weighted least squares fit of the logarithm of sample amount vs number of peptide IDs. Error bars are $\pm$ the standard deviation of the mean.

Figure 4. Normalized selected ion on electropherograms generated at m/z 693.39 (VIELQGIAGTSAAR, a peptide from D-ribose-binding periplasmic protein) from 1, 10, and 100 ng of an *E. coli* tryptic digest. Electropherograms were normalized to unit peak height.