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Dynamic pH junction preconcentration in capillary electrophoresis- electrospray ionization-mass spectrometry for proteomics analysis

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Abstract

Capillary zone electrophoresis (CZE)-electrospray ionization (ESI)-mass spectrometry (MS) is an interesting complimentary technique to reversed phase liquid chromatography (RPLC)-ESI-MS for proteomics research. However, the low sample loading capacity of CZE (typically a few nL) can limit its application for large-scale proteomics. A number of on-line sample preconcentration methods have been developed to increase sample loading volumes. This review considers the dynamic pH junction as a simple on-line sample preconcentration method; this method is well suited for amphiprotic analytes. In the pH junction, these analytes are suspended in a basic buffer, injected by pressure into the capillary, and separated in an acidic background electrolyte, with no changes in either CZE-MS operations or instrumentation. We have demonstrated that the dynamic pH junction method can improve the sample loading volume to sub- μ L volumes without significant loss of separation capacity for bottom-up proteomic analysis. The dynamic pH junction based CZE-ESI-MS system has been applied for a number of complex biological samples, including the *E.coli* proteome, impurities in recombinant antibody therapeutics, and the characterization of the phosphoproteome from a human cell line.

TOC image

Capillary zone electrophoresis (CZE) is a simple method that provides high resolution separation of charged molecules.¹ CZE has proven to be an interesting complimentary

technique to reversed phase chromatography (RPLC) in bottom up proteomic analysis.^{2–4} CZE usually employs a capillary with 50 μm i.d. and a length of up to 1 meter. In conventional CZE, the sample injection volume must be 1% of the total capillary volume to minimize band broadening.⁵ Considering the scale of the capillary, the injection volume is often less than 10 nL, which results in poor concentration detection limits and relatively small numbers of protein and peptide identifications for proteomic analysis. A number of methods have been developed to increase injection volume, including field-amplified stacking, transient isotachopheresis (tITP), dynamic pH junction, sweeping methods, and online solid phase extraction.^{6–8} Of these methods, field-amplified stacking, tITP, and dynamic pH junction are very simple, requiring neither change in instrument setup nor CZE operation.

In field-amplified stacking, the sample is dissolved in a low-conductivity solution and hydrodynamically injected into the capillary, which is filled with a high-conductivity background electrolyte (BGE). Once high voltage is applied across the capillary, the low conductivity portion of the capillary will experience a higher electric field than the rest of the capillary. As a result, the electrophoretic velocity in the sample plug is higher than that in the BGE, which causes stacking of the analyte at the analyte/BGE boundary, which is an efficient preconcentration method for peptides/proteins for CZE-MS.^{4, 9–14} However, there are two limitations of field-amplified stacking when this technique is used on proteomics research. One limitation is low-conductivity solution may cause sample solubility problems. The other limitation is that the maximum injection length of typical field-amplified stacking is limited to about 3–5% of the capillary volume, resulting in a modest enhancement factor.¹⁴

The dynamic pH junction is another simple preconcentration method, which was first reported by Aebersold's group in 1990¹⁵ and named as dynamic pH junction by Britz-McKibbin and Chen in 2000.¹⁶ This technique has been used for a wide range of analytes,¹⁷ and can provide a ten- to a thousand-fold improvement in sample concentration detection limits.

Mechanism of dynamic pH junction

The dynamic pH junction is ideally suited for amphoteric analytes. It is performed by establishing a pH discontinuity in the capillary. In typical operation, analytes are prepared in a basic electrolyte, loaded by pressure into the capillary, and separated in an acidic BGE. Analytes are negatively charged in the basic sample electrolyte. Upon application of a positive potential across the capillary, analytes migrate to the proximal (injection) end of the capillary, where they come in contact with the acidic BGE. Once in the acidic BGE, analytes acquire positive charge and become focused at the boundary between the acidic BGE and the basic sample electrolyte. Once the plug of sample electrolyte is neutralized, analytes are positively charged and migrate to the distal end of the capillary, undergoing conventional zone electrophoretic separation. This form of the dynamic pH junction is popular because high concentrations of acetic acid can be used as a mass-spectrometric compatible BGE. Alternatively, analyte can be prepared in an acidic electrolyte and separated in a basic BGE; this form of the dynamic pH junction is less common for CZE-MS.

Imami and colleagues discussed the focusing mechanism of the dynamic pH junction for a high pH sample electrolyte /low pH BGE using a pH boundary model, where the pH boundary is the boundary between sample matrix and BGE.¹⁸ They demonstrated that the velocity of analyte in the BGE zone in the front of the capillary must be faster than the pH boundary, and velocity of peptides in the sample zone must be slower than the pH boundary. The basic mechanism of the dynamic pH junction was verified by analysis of four peptides prepared in electrolytes prepared at different pH.¹⁸

According to this mechanism, higher pH sample electrolyte will lead to better preconcentration performance when an acidic BGE is used. Hasan and colleagues investigated the effect of the sample electrolyte pH on preconcentration of a peptide with pI 5.5.¹⁹ A solution containing 1 M formic acid at pH 2.0 was used as BGE. Four sample electrolytes (100 mM formate, acetate, bicarbonate, and borate buffers at pH ranges 2.0–4.0, 5.0–6.0, 7.0–8.0, and 9.0–10.0 mixed at 50/50 v/v with acetonitrile) were evaluated. They observed the sensitivity of the CE analysis of peptides increased significantly with an increase in the electrolyte pH for a mixture of model peptides. These results agree well with the pH boundary model. In our research,²⁰ three sample electrolytes were compared for dynamic junction in a bottom-up analysis of the *E. coli* proteome: 10 mM ammonium acetate at pH 6.5, 10 mM NH_4HCO_3 at pH 8.5, and 0.1% $\text{NH}_3\text{H}_2\text{O}$ at pH 10.0. In these experiments, 0.1% formic acid (pH ~ 2.6) was used as BGE. An increase in the pH of the resuspension buffer resulted in higher base peak intensity and sharpening of the peaks generated by late migrating peptides, with the pH 10 sample electrolyte showing the best performance, which is consistent with the pH boundary model mechanism.

In contrast, Ye and colleagues observed a different phenomenon when they analyzed four peptides with similar pI values (5.57, 5.52, 5.52, and 5.52) by using dynamic pH junction CZE-MS with 0.5 mol/L formic acid (pH 2.15) as BGE.²¹ Samples were resuspended in ammonium acetate electrolytes with pH 3.5–9.5. They observed that a sample electrolyte buffer with pH 7.5 instead of pH 9.5 produced the best preconcentration performance for those four peptides. In this report 7.5 mM acetic acid in 80% methanol was used as the sheath buffer, so high pH (9.5) sample matrix may suppress the ionization of sample and cause the lower signal intensity.

Kazarian and colleagues demonstrated that the precise mechanism of the dynamic pH junction can be quite complicated.¹⁷ The preconcentration performance may be provided by more than one mechanism. For example, the use of different ions in the sample and BGE can induce isotachophoretic concentration, field-amplified stacking, or other preconcentration mechanisms. They also provided a comprehensive summary of applications that employed the dynamic pH junction published from 1990 through 2010 that includes the details on the composition and design of the dynamic pH junction systems, such as the composition of electrolyte and sample electrolyte, the pH requirements to induce stacking, analytes of interest, and sensitivity improvement factors.

Two research groups discovered that some stacking from dynamic pH junction is due to tITP.^{22,23} Lunte's group developed a pH-mediated sample stacking method for CE,^{24–26} which is similar to dynamic pH junction. Lunte's group discovered that during pH-mediated

sample stacking, the sample zone is neutralized and produces a low conductivity region wherein analytes are concentrated based on typical field-amplified stacking.²⁶ The mechanism of the pH-mediated sample stacking method also sheds light on the mechanism of the dynamic pH junction method. One recent comprehensive review of moving reaction boundary suggests that the dynamic pH junction method is similar to the moving reaction boundary-induced stacking method.²⁷

Ptolemy *et al.* highlighted advances during 2003–2005 in the dynamic pH junction.²⁸ They described advances in computer simulation that have been used to better understand the mechanism of electrokinetic focusing and also provides a virtual format for in-silico optimization of electrolyte conditions.²⁹

Preconcentration bias of dynamic pH junction

We found that the dynamic pH junction produces a concentration bias during the bottom-up analysis of the *E. coli* proteome.¹⁴ We employed 0.1% formic acid (pH~2.6) as BGE and 10 mM ammonium acetate (pH ~6.5) as the sample electrolyte. In this experiment, we compared the peak-width of several hundred peptides. We observed that the pH junction resulted in a significant decrease in peak-width for all but the fastest and latest migrating components, Figure 1. Components with very short migration time, which tend to be very basic, tend to show no improvement over normal injection conditions. Components with longer migration time, which tend to be strongly acidic, generated wider peaks than conventional injection conditions; those peptides generated wider peaks with lower amplitude in the pH junction experiment and require higher pH sample buffer to undergo pH junction focusing. We verified this hypothesis in a second study,²⁰ when the pH of sample electrolyte was switched to 10. In this case, the peak-width of late migrating peptides tends to be sharper.

Monton and colleagues also found that a dynamic pH junction with low pH sample electrolyte and high pH BGE cannot achieve successful preconcentration for basic peptides. However, using the reverse pH junction technique (high pH sample electrolyte, low pH BGE), produced good preconcentration performance.³⁰

Recently, Chen's group produced a model of concentration efficiency of dynamic pH junction under two modes.³¹ The simulation demonstrates that both dynamic pH junction modes can focus at least 95% of the target molecules injected into the capillary. A longer sample plug could generate a high percentage of molecules captured by dynamic pH junction.

Dynamic pH junction CZE-MS for proteomics research

Proteins and peptides are amphoteric molecules, and will carry a charge in an electrolyte whose pH differs from their pI. Dynamic pH junction can perform outstanding concentration on those compounds. The first dynamic pH junction paper demonstrated the analysis of peptide solutions at five times lower concentration than with normal injection conditions.¹⁵ Using low pH sample electrolyte/high BGE mode, Monton and Imami demonstrated that the dynamic pH junction can produce an improvement in peak height by as much as 124-fold

for two acidic peptides, but no improvement for weakly basic peptides. They further reported that the use of high pH sample electrolyte and low BGE mode, dynamic pH junction can produce up to 1000-fold sensitivity enhancement on cytochrome C digests.^{18,30}

With the development of robust and sensitive CZE-ESI-MS interfaces,^{32–35} CZE is receiving attention as a tool for proteomics research. Preconcentration methods play an important role to increase the sample injection amount and further enhance identification performance of CZE-MS.

In 2014 we first reported the use of the dynamic pH junction based CZE-ESI-MS/MS for bottom-up proteomics with an electrokinetically pumped sheath-flow nanospray CE-MS interface.¹⁴ A linear polyacrylamide coated capillary was used to eliminate electroosmotic flow, which resulted in a longer separation window and a concomitant increase in the number of peptide identifications. As with the BSA digest, the base peak intensity generated by the dynamic pH junction was about 20 times higher than that of the same sample concentration with conventional injection. The comparison of dynamic pH junction and conventional field amplified stacking was also performed. The results show that there is not a significant difference between normal and pH junction injection for short injection length (1 cm). However, the pH junction produced superior results for a larger injection volume (10 cm). There are limits to the sample volume that can be used in the pH junction before peak broadening occurs. We observed a linear increase in peak height vs. injection volume up to an injection volume of 250 nL, which is 25% of the capillary volume. Larger sample volumes generated broader peaks, which degrades the separation efficiency. We also reported the superior preconcentration performance of three standard intact proteins (cytochrome C, myoglobin, and beta casein) with a pI from 5 to 10, which suggests that the dynamic pH junction may be of value in top-down proteomics research.¹⁴

The dynamic pH junction is also useful in the automation of CZE-MS. In 2014, we reported a stable, reproducible, and automated CZE-MS system with our electrokinetically pumped sheath-flow nanospray interface.³⁶ In that report, the sample was dissolved in 25% acetonitrile solution to perform conventional field amplified injection. Due to the volatility of sample electrolyte, the concentration of acetonitrile and the volume of sample electrolyte changes in the sample tray during sample analysis, and we only succeeded in generated eight sequential runs of the *E. coli* digest. In contrast, the sample can be dissolved in nonvolatile electrolyte in the dynamic pH junction, which improves long-term operation. In 2016 we combined dynamic pH junction sample preconcentration with an improved CE-MS interface to generate 97 consecutive separations of a BSA tryptic digest over a 145-h period.³⁷ Separation efficiency averaged over 100,000 theoretical plates across this period. With this robust automated CZE-MS system, we analyzed trace amounts of host cell protein impurities in recombinant therapeutics using both the dynamic pH junction CZE-MS and RPLC-MS.²⁰ Both CZE-MS/MS and RPLC-MS/MS consistently detect peptides from spiked impurity proteins present at the 100-ppm level in the presence of 1 mg/mL recombinant antibody. However, CZE-MS/MS generated approximately five times higher base peak intensity and more peptide identifications for low-level spiked proteins than RPLC-MS/MS. Linear calibration curves were also obtained by this stable and reproducible CZE-MS system, and the linearity is comparable to RPLC-MS. These results suggest that

dynamic pH junction based automated CZE-MS is a stable and robust tool for label free quantification.

Dynamic pH junction based CZE-MS/MS also showed the powerful identification capability of an enriched phosphoproteome from the MCF-10A cell line.³⁸ A total of 2,313 phosphorylated peptides were identified with single-shot CZE-MS/MS in a 100 min analysis with about 1 μ g sample loading amount. This number of phosphorylated peptide IDs is over one order of magnitude higher than the number of phosphorylated peptide IDs previously reported by single-shot CZE-MS/MS. Single-shot nanoRPLC-MS/MS produced around 3,300 phosphorylated peptide IDs from 2 μ g of enriched phosphoproteome of the MCF-10A cell line. When the sample loading amount is in low ng range, CZE-MS/MS can generate significantly more phosphopeptide IDs than nanoRPLC-MS/MS. Additionally, CZE-MS and nanoRPLC-MS are complimentary for phosphorylated peptide IDs. It is worth noting that CZE has significant better performance than RPLC for resolving phosphorylated and non-phosphorylated peptide pools.³⁸ Phosphorylation of peptides results a decrease in a peptide's charge, which significantly changes the migration time of those peptides. Faserl *et al.* demonstrated that phosphopeptides migrate significantly slower than most of other peptides present in the sample in CZE-MS.³⁹ This feature makes CZE-MS very useful for peptide post-translational modifications (PTMs) analysis because it can help to reduce the interference of modified peptide identification from non-modified peptides. Due to the very simple separation mechanism of CZE, the accurate prediction of migration time of peptides is relatively simple in CZE compared to RPLC. This feature makes CZE very useful for peptide identification with MS based on the accurate migration time and m/z of peptides. In addition, the accurate prediction of migration time of peptides can also be valuable for assisting the database searching of MS/MS to provide more confident peptide identification, especially for peptides with post-translational modifications (PTMs).

Dynamic pH junction can also be coupled with other preconcentration methods. For example, our group prepared a sulfonate-silica hybrid strong cation-exchange (SCX) monolith at the proximate end of a CZE column to perform on-line solid-phase extraction (SPE) sample preconcentration, as shown in Figure 2.⁴⁰ In this work, protein digests were dissolved in an acidic buffer and concentrated onto the SCX-SPE monolith. Peptides were then eluted by pressure into an acidic BGE using a relatively large (~100 nL) plug of basic electrolyte. Electrophoresis produced narrow peaks as a result of the pH junction produced in this experiment. This technique was applied for the analysis of diluted *E.coli* digests. By loading 5.5 μ L of a 10^{-3} mg/mL (5.5 ng) *E. coli* digest, 109 proteins and 271 peptides were identified in a 20 min separation. This technique was further improved to perform online fractionation of complex proteome digests followed by CZE-MS/MS analysis, and ~800 protein groups were identified from 50 ng of *E.coli* proteome digest in 2.5 h instrument time.⁴¹

Besides proteomic applications, dynamic pH junction based CZE-MS has also been used for analysis of metabolites. Lee *et al.* employed dynamic pH junction based CZE-MS for online concentration and cleanup of the low abundant metabolites, and obtained 50-fold improvement of sensitivity for cationic metabolites. They integrated this platform and computer simulations for studying the selective nutrient uptake behavior of *E. coli*.⁴² In that

work, Lee *et al.* also demonstrated that the dynamic pH junction based CZE has the great potential for online desalting of biological samples, and this feature will be invaluable for improving the CZE-MS sensitivity for analysis of real biological samples due to the reduced sample loss during sample cleanup and also the reduced ion suppression during MS analysis.⁴²

Conclusion

The dynamic pH junction is an ideal preconcentration method for amphiprotic analyte, such as peptides produced in bottom-up proteomics and proteins determined in top-down proteomics. The technique simply requires that analytes are suspended in a basic electrolyte, and pressure injected into an acidic BGE. Application of electric field automatically results in the concentration and subsequent electrophoretic separation of analytes. The technique is now commonly used in our group and has been demonstrated for analysis of a wide range of complex proteomes.

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Figure 1.

Peak width, defined as the standard deviation of the Gaussian function used to fit the electrophoretic peak, is plotted versus migration time for a 20 nL injection of a 0.1 mg/mL *E. coli* tryptic digest under normal and pH junction conditions.¹⁴