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Optimization of dynamic pH barrage junction focusing for weakly alkaline or zwitterionic analytes in capillary electrophoresis

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ABSTRACT

Dynamic pH junction focusing prior to electrophoretic separation has been widely used for online pre-concentration of biologically important analytes, which are mostly weakly alkaline/acidic or zwitterionic species such as neurotransmitters, peptides, and proteins. A pH junction is formed when background electrolytes with different pH values are injected sequentially into the separation column of a capillary electrophoresis (CE) system. Unlike the traditional dynamic pH junction configuration with analyte molecules located in a different chemical environment to the separation background electrolyte (BGE), the pH barrage junction has a separate high pH (or low pH) region containing no analyte. Based on *Simul 5 Complex* simulations and experimental verification with three series of electrolyte combinations, four basic principles for pH barrage junction focusing were identified for its optimization. First, the peak shape after focusing is slightly asymmetric, but this has negligible influence on the analysis result. Second, longer length of the barrage segment is needed for complete focusing with lower concentration of the buffering species. Third, this technique is more advantageous for analytes with relatively high electrophoretic mobility in a capillary without electroosmotic flow. Fourth, provided the analyte region and pH junction buffering species are separated, this quantitative technique is compatible with both optical and mass spectrometric detection.

1. Introduction

Capillary electrophoresis (CE) is an analytical technique that uses very small volume of samples, which is advantageous in the analysis of biological samples with limited quantities. To achieve good separation of adjacent peaks, the use of a short sample plug, which is usually less than 1% of total separation capillary length, is generally recommended, meaning that less than 10 nL of sample is usually injected for a CE analysis. Despite the method's low mass detection limit, it requires a relatively high analyte concentration because of the detector's short light path, which is equal to the inner diameter of the capillary if on-column detection is used [1]. Analytes in biological samples are usually much too low in concentration to be detected even with off line enrichment procedures. Several methods have been used to overcome the need for high analyte concentrations [2–5]. In addition to various off-line analyte pre-concentration strategies, there have been efforts to use alternative detection technologies that are compatible with CE, such as laser-induced fluorescence (LIF) and state-of-the-art mass spectrometry (MS) [6–8]. Limit of detection (LOD) of a single molecule can be achieved with LIF, and LOD of circa 600 peptide molecules sensitivity can be achieved with MS [8–10]. Another strategy is to use online pre-concentration processes based on the physicochemical properties of the separation BGE such as its conductivity [3], or the surface affinity of the analyte, which is exploited in online SPE methods [11]. Another feasible alternative is to use a pH-mediated stacking technique such as dynamic pH junction focusing [12–14], acid/base induced stacking [15,16], moving reaction boundary stacking [17], moving neutralization boundary focusing [18], and transient moving chemical reaction boundary method [19].

Conventional CE requires the sample composition to be as similar to the separation BGE as possible, to avoid pH/conductivity-induced peak distortions. Weakly alkaline/acidic analytes can be selectively focused by adjusting the pH of the sample segment and using long sample injections because of the different velocities of such analytes at different pH environments [12,13]. By increasing the sample injection volume to 30% of the separation capillary volume, the analyte signal can be

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enhanced by up to two orders of magnitude, enabling the analysis of bio-active species with extremely low concentration, such as nucleotides and catecholamines. Recently, microliter-level sample loading capacity had been achieved in CE-based proteomic analysis with both top-down and bottom-up approaches [20,21]. Simulations have established the formation of a moving dynamic pH boundary and a quasi-stationary one, which together sandwich the long sample segment and selectively focus the analyte into a narrower zone with much higher concentration [14,22,23]. The dynamic pH boundary is the key to successful analyte focusing, but the location of pH junction relative to the sample region can be different. By placing a high pH segment between the acidic separation BGE and the acidic sample solution of the weakly alkaline analyte, similar results can be obtained based on the differences in analyte electrophoretic mobility between the separation BGE and high pH barrage region, which results from the analyte deprotonation. In this scenario, the pH barrage segment works as a barrier to stop and stack analyte molecules.

This technique can also be regarded as a form of dynamic pH junction focusing, although some publications have described this arrangement as acid barrage focusing [24,25], and two similar methods have been referred to as the acid/base induced stacking technique interpreted from the aspect of transient isotachophoresis (tITP) [15,16]. A review by Kitagawa and Otsuka [3] described the recent use of similar pH-mediated stacking methods for the analysis of various biological samples, including determining or quantifying catecholamine in high ionic strength samples [26], organic acid anions in physiological samples [27], small and medium-sized peptides [28], derivatized amino acids in high ionic strength solutions and native amino acids in urine samples [24,29], fumaric acid and maleic acid in apple juice [30], opiate-related compounds in saliva [31], and drugs of abuse in urine [32]. Both electrokinetic and hydrodynamic injection can be used for the different analyte types listed above.

Although the studies listed above have used the pH barrage junction focusing technique to achieve considerable signal enhancement, there have been no comprehensive investigations into the mechanism details of this focusing technique. To facilitate the adoption of this method in the analyst/biologist community, its overall focusing–separation optimization for specific analyte, means for improving resolution using different barrage length, and the reasons this stacking technique can be used for quantification, need to be understood. Here we present a study with simulations and experiments in addressing these questions. Simulations were performed in silico using *Simul 5 Complex* [33], and CE bench experiments for verification of theoretical predictions were performed with either optical absorbance detection or mass spectrometric detection.

2. Materials and methods

2.1. Theoretical study using Simul 5 Complex

Twenty-six simulations were performed using three desktop computers equipped with Intel Core i5/i7 CPUs. Real-world parameters were adopted as much as possible, but the simulations were limited by the computation resource available. Therefore, we shortened the simulated separation capillary by a factor of 24 relative to that used in real experiments (20 mm versus 48 cm, total capillary length). It allowed us to perform simulations at a resolution that provided insight into the mechanism details with acceptable computational costs. Using this approach, individual simulations generally took 12 to 20 h. The simulation settings are listed in Table 1.

Nicotine was used as a dibasic model analyte in both the simulations and the real experiments, and its pK_a values were 3.10 and 8.01 [34]. The separation BGE was formic acid (FA, pKa 3.75), and the pH barrage was a buffer consisting of NH_3 and FA in a 10:1 molar ratio (100 mM NH_3 with 10 mM FA, 200 + 20 or 300 + 30, pH of 10.2). Nicotine was largely positive in the barrage segment. The lengths of the sample and barrage segment were set to achieve similar length ratios to those used in real experiments. Due to the diffusion phenomenon and the parabolic flow pattern during the pressure-driven sample injection, distributions of all species over the capillary were initialized with continuous regions of 0.05 mm (peak edge width) to account for concentration changes, instead of sudden drop/rise with no smooth transitions.

2.2. Focusing and separation of nicotine and a 5-peptide mixture with a UV detector

A P/ACE MDQ automated CE instrument and a PA800+ CE instrument from Sciex Separations (Brea, CA, USA) were used for all CE–UV analyses. A 48-cm capillary (Polymicro Technologies, Phoenix, AZ, USA) with a 50-μm inner diameter was used for focusing and separation of nicotine (ACS grade, Fisher Scientific, Burlington, ON, Canada) and a 5-peptide mixture sample (Agilent H2016-1VL peptide standard, Fisher Scientific). The effective length of the capillary was 38 cm and the total length was 48 cm. Hydroxyl-propyl cellulose 5% w/w (HPC, 10,000 Mw, Fisher Scientific) was used to coat the capillary to eliminate EOF following a reported protocol [35]. The separation voltage was 20 kV, and the BGE contained no HPC additives. Because of the high electrophoretic mobility of nicotine and NH_4^+, it was not necessary to apply any pressure to accelerate the separation, which was completed in less than 6 min. However, a pressure of 0.5 psi was applied when performing peptide analyses to accelerate the analysis. The BGE was 200 mM formic acid (88%, Fisher Scientific) in water, and pH barrage buffer was 100:10, 200:20, 300:30, NH_3 (mM) to FA (mM). The detection wavelength was 214 nm for both nicotine and peptides. The sample segment length and the barrage segment length were controlled empirically by the injection time under 1.0 psi (e.g., 28 s for 10% of the effective capillary length).

2.3. Focusing and separation of a 5-peptide mixture with a quadrupole time-of-flight mass spectrometer (Q-TOF MS) detector

The flow-through microvial CE–MS interface was used to couple CE to a Micromass Q-TOF MS (Waters, Milford, MA, USA) [36,37]. A 60-cm bare fused silica (BFS) capillary with a 50 μm inner diameter was used for separation, and the effective separation length was the same as the total capillary length. The BGE was 200 mM FA with 30% methanol (v/v, MeOH, LC–MS grade, Fisher Scientific). The chemical modifier was 200 mM FA with 75% methanol (v/v), with a flow rate of 200 nL/min. In this strongly acidic environment, the EOF of the BFS capillary was suppressed. Separation was driven using a potential of 30 kV and a pressure of 0.5 psi was utilized to shorten the analysis time. Since the electrospray (ESI) voltage was 4.0 kV, the effective voltage for separation was 26 kV. The pH barrage consisted of 200 mM ammonia (diluted from the stock solution of NH_3, 28%, 15.3 M, Fisher Scientific) with 20 mM FA in 30% methanol (v/v) and its injection was 1 psi for 20 s. The sample length of 10% capillary effective length required 59 s injection at 1 psi, while in CZE it was 1 psi for 5 s. Water with the resistance of 18 MΩ/cm was used to prepare all aqueous solution, and it was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

2.4. Calibration curve for nicotine after focusing with a triple quadrupole mass spectrometer (QqQ MS)

A Sciex API 4000 QqQ MS (Sciex, Concord, ON, Canada) was used in multiple reaction monitoring (MRM) mode. The same micro-vial CE–MS interface was used to interface the Beckman PA 800+ automated CE to the QqQ MS. The separation capillary was 66 cm long and was coated with polyethylene imine (PEI) for a fast, reversed EOF with acidic BGE [38]. The BGE was 200 mM FA with 30% methanol (v/v), and the modifier was 200 mM FA in 75% methanol (v/v), with a flow rate of 200 nL/min. The pH barrage was 100 mM ammonia and 10 mM FA in 30% methanol (v/v) and its injection was 1 psi for 48 s. The