On-Line Sample Preconcentration Using Field-amplified Stacking Injection in Microchip Capillary Electrophoresis

Maojun Gong,† Kenneth R. Wehmeyer,‡ Patrick A. Limbach,§ Francisco Arias,∥ and William R. Heineman*,†

Department of Chemistry, University of Cincinnati, P.O. Box 210172, Cincinnati, Ohio 45221-0172, and Procter and Gamble Pharmaceuticals, Health Care Research Center, 8700 Mason-Montgomery Road, Mason, Ohio 45040

Previous reports describing sample stacking on microchip capillary electrophoresis (μCE) have regarded the microchip channels as a closed system and treated the bulk flow as in traditional capillary electrophoresis. This work demonstrates that the flows arising from the intersection should be investigated as an open system. It is shown that the pressure-driven flows into or from the branch channels due to bulk velocity mismatch in the main channel should not be neglected but can be used for liquid transportation in the channels. On the basis of these concepts, a sample preconcentration scheme was developed in a commercially available single-cross glass chip for μCE. Similar to field-amplified stacking injection in traditional CE, a low conductivity sample buffer plug was introduced into the separation channel immediately before the negatively charged analyze molecules were injected. The detection sensitivity was improved by 94-, 108-, and 160-fold for fluorescein-5-isothiocyanate, fluorescein disodium, and 5-carboxyfluorescein, respectively, relative to a traditional pinched injection. The calibration curves for fluorescein and 5-carboxyfluorescein demonstrated good linearity in the concentration range (1–60 nM) investigated with acceptable reproducibility of migration time and peak height and area ratios (4–5% RSD). This preconcentration scheme will be of particular significance to the practical use of μCE in the emerging miniaturized analytical instrumentation.

Microchip capillary electrophoresis (μCE) offers several advantages over traditional capillary electrophoresis (CE), such as reduced sample and reagent consumption, short analysis time, and easy miniaturization of analytical instrumentation; however, the small sample injection volume and short path length available for optical measurements limit the detection schemes of μCE even more than in traditional CE. To improve sensitivity for UV detection, z- or u-shaped optical path and multireflection cells have been employed in μCE. Laser-induced fluorescence (LIF) detection instead of UV absorbance provides greater sensitivity in both traditional CE and μCE. An additional means of improving detection limits in CE and μCE is to employ sample pretreatment schemes. The most widely adapted approaches for on-line sample pretreatment include isochromatography, solid-phase extraction, and, recently, sweeping techniques. Sample stacking is widely used in traditional CE in which detection enhancement factors from 10 to 1000 have been achieved. In a typical sample stacking approach for anions, the analyte in a low-conductivity sample buffer plug (SBP) is injected into the capillary inlet by pressure, and then a normal-polarity voltage is applied across the capillary. The sample anions become stacked at the interface between the low- and high-conductivity buffers due to the higher electric field strength in the low-conductivity buffer driving faster migration of the anions.


* Corresponding author. Tel: (513) 556-9210. Fax: (513) 556-9239. E-mail: William.Heineman@uc.edu.
† University of Cincinnati.
§ P&G.

10.1021/ac0521798 CCC: $33.50 © 2006 American Chemical Society Published on Web 05/03/2006
relative to the running buffer. However, the laminar flow profile caused by hydraulic pressure on the buffer gradient boundary results in band-broadening and loss of resolution. Therefore, the injected SBP length is typically kept to ~10% of the effective capillary length so as to achieve a satisfactory compromise between resolution and enhanced detection sensitivity.

To achieve higher detection sensitivity while maintaining high resolution, field-amplified stacking injection (FASI) has been developed and used in traditional CE. For an anionic analyte, FASI is achieved by introducing a low-conductivity solution, such as water, into the capillary from the inlet by pressure. The capillary inlet is then inserted into the sample solution, and a reversed-polarity voltage is applied across the capillary. Because of the higher electric field strength in the water plug relative to the running buffer, analyte anions are injected and stacked at the buffer boundary, and simultaneously, the water plug is pumped out of the capillary by the EOF induced by the electric field. This method can afford a sensitivity enhancement of several thousand-fold in traditional CE.

FASI cannot be directly transferred to μCE due to the complexity of the injection and separation channel design. In the simplest μCE design with a single cross, there are four branch channels meeting at the intersection. One set of branch channels comprise the separation capillary (usually channels L2 and L4), and the other set of channels comprise the injection capillary (usually channels L1 and L3), as shown in Figure 1. The existence of these branch channels creates an open system and makes the practice of sample stacking more complicated. Additionally, the specialized “pinched” injection used with μCE involves multiple voltage steps and demands the application of properly balanced voltages to the reservoirs to avoid diffusion problems.

The first reported sample stacking on a single-cross fused-quartz microchip was performed by Jacobson and Ramsey using a modified gated injection scheme suitable for negatively charged species with low mobility. Their approach resulted in an ~10-fold detection enhancement, which was limited by the pressure-driven peak broadening effects.

In recent years, two additional sample stacking schemes on μCE devices have been developed. One approach is to inject a large volume of a low-conductivity sample solution into the separation channel, or an additional branch channel, and then simultaneously push sample buffer out of the separation channel while stacking the analytes. The other approach is to stabilize the conductivity gradient boundaries in the injection region by using an additional branch channel or a porous polymer structure.

Although these two sample stacking approaches can increase detection sensitivity up to 1000-fold, they are limited by more complicated microchip designs, laborious analytical procedures, a poorly controlled sample injection volume, and difficulty in knowing the locations of preconcentrated analytes of interest.

In this paper, preconcentration of anionic analytes was achieved using a SBP injection, followed by FASI with sample buffer removal from the separation channel on a commercially available single-cross glass microchip. The SBP injection and FASI processes were theoretically and experimentally studied, and the stacking process was monitored at different locations along the separation channel using anionic and neutral dyes. The optimal injection and separation conditions were developed with fluorescein-5-isothiocyanate (FITC), fluorescein disodium salt (FL), and 5-carboxyfluorescein (5-FAM). Finally, the preconcentration scheme was evaluated by examining detection enhancement relative to the standard pinched injection and by constructing calibration curves for FL and 5-FAM using FITC as an internal standard.

EXPERIMENTAL SECTION

Instrumentation. A custom-built μCE system described previously was used for all analyses. The analysis was performed on a commercially available (Micralyne Inc., Edmonton, AB, Canada) single-cross glass microchip (Figure 1). The chip has separation and injection channel lengths of 8.50 and 0.80 cm, respectively. Polypropylene reservoirs were attached at the sample points.

Figure 1. Four-step procedure for sample buffer plug (SBP) injection/field-amplified stacking injection (FASI). (a) Loading: the low-conductivity sample solution is pumped through the channel intersection into the SBP until the flow reaches a steady state. (b) SBP injection: a SBP is pumped into the separation channel; simultaneously, the sample buffer solution also flows into channels L2 and L3 and accumulates in the BR and WB reservoirs, respectively. (c) FASI: charged analytes from channel L1 are introduced into the separation channel, and simultaneously, the SBP is pumped backward into SW. (d) Dispensing/separation: analyte zones are separated from one another and are detected with LIF. SR = sample reservoir; SW = sample waste reservoir; BR = buffer reservoir; WB = buffer waste reservoir; F = floated; G = grounded. Unit of voltages beside each reservoir is kilovolt (kV). L1–4 are lengths of four channels from the intersection. L5 is the sample buffer plug length in channel L5. Arrows indicate bulk flow directions. Size is not proportional to the real dimensions.

reservoir (SR), the sample waste (SW), the buffer reservoir (BR), and the buffer waste (BW) using a two-part epoxy. The filtered fluorescence excitation beam (480 ± 20 nm) from a 150-watt xenon lamp was focused onto the microchip using the 20× objective of an inverted microscope (Nikon Eclipse TE300, Nikon Corp., Melville, NY), and the emission was spectrally and spatially filtered using a 525 ± 25 nm band-pass filter and a 1-mm pinhole, respectively. In addition, a CCD-100 camera system (Dage-MTI Inc., Michigan City, IN) was coupled to the bottom of the microscope and was used together with a video monitor (model BWMC, Javelin Systems, Torrace, CA) to record and quantitatively observe flows and analyte movement in the microchip channels.

**Chemicals and Reagents.** Sodium tetraborate and sodium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ). FL was from ICN Biomedicals, Inc. (Aurora, OH). BODIPY 530/550 (4,4-difluoro-5,7-diphenyl-1-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester), BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) and FITC isomer I were from Molecular Probes (Eugene, OR). 5-FAM was from Aldrich Chemical Co., Inc. (Milwaukee, WI). All chemicals were used without further treatment.

BODIPY stock solutions were prepared with methanol, and all other solutions were prepared with deionized water from a NANOpure system (Sybron Barnstead Corp., Boston, MA). FITC, FL, and 5-FAM stock solutions were prepared in water at concentrations of 0.25, 1.0, and 0.44 mM, respectively, and were diluted with water, buffer, or both as needed. All solutions used in filling the chip reservoirs were filtered through 0.2-μm syringe filters purchased from Gelman Laboratory, Pall Corp. (Ann Arbor, MI), and the buffer and sample solutions were degassed under vacuum.

**μCE Procedures.** Chip Conditioning. The channels of the glass chip were conditioned by filling the SR, BR, and SW reservoirs and washing sequentially for 10 min each with 1.0 M NaOH, deionized water, and running buffer by applying a vacuum to BW. After the NaOH rinse, all reservoirs were flushed several times with deionized water.

**SBP and FASI Process.** Following conditioning, an aliquot (30 μL) of running buffer (35.0 mM tetraborate, pH 8.9) was added to BW and SW, 35 μL of running buffer was added to BR, and an aliquot (30 μL) of the analyte in the sample buffer solution (0.50 mM tetraborate, pH 8.3 buffer, unless indicated otherwise) was added to SR. After focusing the detection point of the laser on the main channel, the Labview and Turbochrome programs were manually switched on simultaneously using the voltage setup shown in Figure 1 for the SBP injection/FASI procedure or for a standard pinched injection. All experiments were performed at ambient temperature. All data were processed with Turbochrome software (Version 4.0). Flow videos were captured by the CCD camera and recorded on a videotape, and the flow pictures were selected from the video.

**EOF and Electrophoretic Mobility Determination.** The EOF and the electrophoretic mobility of FL, FITC, and 5-FAM were determined using the same running buffer and sample buffer configuration discussed above in conjunction with a pinched injection program and BODIPY 530/550 as a neutral marker. The EOF was determined from the migration time of the neutral marker, and the electrophoretic mobility of each analyte was then determined.36

**RESULTS AND DISCUSSION**

**Description of the SBP and FASI Process.** On the basis of the FASI mechanism used in traditional CE, 24-26 a FASI scheme for stacking in μCE was developed. A four-step procedure was designed as shown in Figure 1 using the SBP/FASI voltage configuration shown in each step. First, the low-conductivity sample solution is electrosotomically pumped from SR through the intersection into the SW reservoir until the flow reaches a steady state. Then, the voltage setup is switched to the SBP injection state, with the application of the voltage between SR and BW resulting in the sample buffer solution's being pumped from SR and driven into channels L0, L6, and L4. Next, for FASI, the voltage polarity is reversed from the SBP injection, with the voltage being applied between SW and BW resulting in bulk flow into SW through L0. This bulk flow results in siphoning flows from SR, BR, and BW through L1, L6, and L4, respectively, then the intersection and into SW via L6. During the FASI step, analyte anions from SR enter the intersection via L1 and are accelerated and introduced into the separation channel (L0) due to the applied voltage between SW and BW. The analyte anions are stacked at the interface between the low- and high-conductivity buffers in L4, and simultaneously, most of the low-conductivity buffer is pumped out of L4 into the SW reservoir. Finally, at the appropriate time, the voltage setup is switched to the separation/dispensing step just before the concentrated sample arrives at the intersection, and the analyte zones are separated from one another and detected. Steps 1 and 4 in this process are the same steps used for a pinched injection in μCE and will not be further considered. The SBP injection (step 2) and FASI (step 3) are more complicated and will be discussed in detail below.

**Theoretical Considerations for SBP Injection.** Due to the complexity of the channels in the simple cross microchip used here, the SBP injection and FASI process are more involved than in traditional CE. During the SBP injection step, electrodes for BR and SW are floated, BW is grounded, and SR has a voltage of U0; thus, the voltage is applied only between SR and BW (Figure 1). Due to the mismatch of EOF velocities produced in L1 and L4 as a result of the difference in buffer conductivities, the bulk flow from L4 (V4) is divided into three flows into L6, L0, and L4 with bulk velocities of V6, V0, and V4, respectively. Due to the fluid continuity, the solution volume into the intersection is equal to the total volume out as described by eq 1, where A1, A2, A3, and A4 are the cross-sectional areas of the four channels.

\[
V_1A_1 = V_2A_2 + V_3A_3 + V_4A_4 \quad (1)
\]

For simplicity, suppose \(A_1 = A_2 = A_3 = A_4\); then

\[
V_1 = V_2 + V_3 + V_4 \quad (2)
\]

At the moment \(t_0 = 0\) of voltage switching from the loading step to the SBP injection, L4 is full of sample buffer, L6 and L4 are full of running buffer, and L6 is filled with a mixture of sample buffer and running buffer (Figure 1a and b). Electric field

strengths across L₁ ({E_{10}}) and L₄ ({E_{40}}) are described by eqs 3a and 3b, respectively,

\[
E_{10} = \frac{\gamma U_0}{\gamma L_1 + L_4} \tag{3a}
\]
\[
E_{40} = \frac{U_0}{\gamma L_1 + L_4} \tag{3b}
\]

where \( \gamma \) is the conductivity ratio of the running buffer plug (RBP) and the SBP, and \( U_0 \) is the applied voltage between SR and BW. After time \( t_x \), a SBP with the length of \( L_x \) is injected into L₄ as shown in Figure 1b where \( x \) refers to the position of the SBP front in L₄ relative to the intersection. Then, the electric field strengths across the SBP (\( E_{1x} \)), which is across \( L_1 + L_x \), and across the RBP (\( E_{4x} \)), which is across \( L_4 - L_x \), are described by eqs 4a and 4b, respectively.

\[
E_{1x} = \frac{\gamma U_0}{\gamma (L_1 + L_x) + (L_4 - L_x)} \tag{4a}
\]
\[
E_{4x} = \frac{U_0}{\gamma (L_1 + L_x) + (L_4 - L_x)} \tag{4b}
\]

Accordingly, bulk flows (\( V_{ix} \)) in each of the branch channels (\( L_1, L_2, L_3, \) and \( L_4 \)) are the vector sums of the local electroosmotic velocities and the pressure-driven velocities (\( V_{ip}, i = 1-4 \)) in each channel \( L_i \), as expressed in eqs 5a–d.

\[
V_{1x} = V_{e01x} - V_{p1x} \tag{5a}
\]
\[
V_{2x} = V_{p2x} \tag{5b}
\]
\[
V_{3x} = V_{p3x} \tag{5c}
\]
\[
V_{4x} = V_{e04x} + V_{p4x} \tag{5d}
\]

where \( V_{e0ix} \) and \( V_{e04x} \) are local EOFs produced in L₄ and L₄, respectively. The pressure-driven velocity is produced by the imbalance of local EOFs in the low-conductivity sample buffer plug and the running buffer.\(^{2,27} V_{e0ix} \) can be expressed in terms of the local electroosmotic mobility (\( \mu_{e0i} \)) and electric field strength (\( E_{0i} \)), as shown in eq 6.

\[
V_{e01x} = \mu_{e01} E_{1x} \tag{6}
\]

Since the \( L_x \) part of L₄ is occupied by sample buffer and the rest of L₄ is still full of running buffer, \( V_{e04x} \) is the average of two parts weighted by the ratios, as described by eq 7.\(^{23,38} \)

\[
V_{e04x} = \left( \frac{L_4}{L_x} \right) \mu_{e01} E_{1x} + \left( 1 - \frac{L_4}{L_x} \right) \mu_{e04} E_{4x} \tag{7}
\]

where \( \mu_{e0i} \) is the electroosmotic mobility in the running buffer.

From eqs 2 and 5,

\[
V_{e01x} - V_{p1x} = V_{p2x} + V_{p3x} + (V_{e04x} + V_{p4x}) \tag{8}
\]

If it is assumed that the fluid viscosities in the four channels are the same, the chip sits on a horizontal plane, and the reservoirs contain the same level of liquid, then the pressure-driven flows will be inversely proportional to their channel lengths, as expressed in eq 9.\(^{37} \)

\[
L_1 V_{p1x} = L_2 V_{p2x} = L_3 V_{p3x} = L_4 V_{p4x} = b \Delta P \tag{9}
\]

where \( \Delta P \) is the pressure drop between the channel end and the intersection, and \( b \) is a constant. From the combination of eqs 8 and 9, pressure-driven flow velocities are obtained as shown in eq 10.\(^{37} \)

\[
V_{pax} = -\frac{\Delta V_{e0x}}{L \left( \frac{1}{L_1} + \frac{1}{L_2} + \frac{1}{L_3} + \frac{1}{L_4} \right)} \quad i = 1 - 4 \tag{10}
\]

where \( \Delta V_{e0x} = V_{e01x} - V_{e04x} \) is the difference in EOFs in channels L₁ and L₄, and it can be expressed as the function of \( L_x \) derived by combining eqs 4, 6, and 7.

\[
\Delta V_{e0x} = \frac{U_0 (\gamma \mu_{e01} - \mu_{e04})(1 - L_x/L_4)}{\gamma (L_1 + L_2) + (L_4 - L_x)} \tag{11}
\]

Generally, \( \gamma \mu_{e01} \) is much larger than \( \mu_{e04} \) when the two buffers have similar pH values, and as a result, \( \Delta V_{e0x} \) is positive, and the SBP in channel L₄ produces a pressure to the solution plugs in the branch channels connected to the intersection.\(^{37,39,40} \)

For a specific model system on a microchip, the applied voltage and the buffer conductivity ratio are selected and kept constant, assuming that electrolysis, ion diffusion, and liquid convection are negligible. In our case, \( U_0 = 1000 \) V and \( \gamma = 70 \) (ratio of the tetraborate concentrations of the running buffer and the sample buffer); \( L_1 = L_3 = 0.40 \) cm, \( L_2 = 0.50 \) cm, and \( L_4 = 8.00 \) cm. The electroosmotic mobilities for 0.50 and 35.0 mM tetraborate buffer are \( 6.18 \times 10^{-4} \) and \( 4.06 \times 10^{-4} \text{cm}^2/\text{V} \text{s} \), respectively. Substituting these values into eq 11, the expressions of \( \Delta V_{e0x} \) and \( V_{pax} \) (eqs 5a–d, \( i = 1-4 \)) were obtained in terms of sample buffer plug length \( L_x \) in the separation channel. The relationship diagrams for \( \Delta V_{e0x} \) and bulk channel velocities versus \( L_x \) are shown in Figure 2. As can be seen, \( \Delta V_{e0x} \) is decreasing rapidly as the SBP is injected into L₄. Accordingly, the bulk flow velocities in channels L₁, L₂, and L₃ are decreasing; however, the bulk flow velocity in L₄ is still gently increasing, which is caused by the higher electroosmotic flow velocity of the low conductivity sample buffer injected into L₄. It also shows that the bulk velocities in L₂ and L₃ (\( V_{p2x} \) and \( V_{p3x} \)) are significantly higher than that in L₄ (\( V_{p4x} \)) when \( L_x \) is short. It is easy to understand that the pressure-driven flow velocity in L₄ is significantly lower than in L₂ and L₃, since the lengths of


(38) Chien, R.-L.; Burgi, D. S. Anal. Chem. 1992, 64, 489A–496A.


the electroosmotic velocity, $V_{eo}$, is relatively low due to the high conductivity of the running buffer relative to the sample buffer.

The bulk flows from the intersection during the SBP injection were monitored with a neutral dye, BODIPY 505/515. As can be seen in Figure 3a, as soon as the SBP injection voltage was switched on, the SBP was introduced into $L_4$ as well as into $L_2$ and $L_3$. After 100 ms, the SBP length in $L_4$ is ~4 times shorter than that in $L_2$, as predicted by the relative bulk flow velocities described in Figure 2 and, thus, provides visual support for the validity of the calculations. As time progresses, the neutral dye migrates further into channels $L_2$ and $L_4$. It should be noted channel $L_3$ is filled with the neutral dye at all times during the SBP injection, since it was introduced into this channel during the sample loading step (Figure 1a).

The movement of an anionic dye was also visually observed. Under the applied voltage, the negatively charged analyte in the sample buffer has an electrophoretic velocity, $V_{ep}$, of the analyte anions can be expressed as in eq 12.

$$V_{ep} = \frac{\mu_{ep} \gamma U_0}{\gamma (L_4 - L_2)}$$  \hspace{1cm} (12)

By inserting the values of all the variables ($L_4$, $L_2$, $U_0$, and $\mu_{epFL} = 1.46 \times 10^{-4}$ cm$^2$/Vs in 0.50 mM tetraborate) into eq 12, the electrophoretic velocity of FL anions ($V_{epFL}$) in the low-conductivity buffer using 35.0 mM sodium tetraborate as the high-conductivity buffer can be obtained. The apparent velocity of the FL anions in the SBP of $L_3$ is the vector sum of the bulk flow $V_{B}$ (eq 5d) and the electrophoretic velocity $V_{epFL}$ (eq 12). When $V_{epFL} > V_{B}$, i.e., $L_2 \leq 1.70$ cm, the FL anions are moving toward the intersection and are rejected from entering the separation channel. This effect is confirmed by monitoring the FL anions during the SBP injection process (Figure 3b). It can also be seen in Figure 3b that FL anions do not enter $L_3$ but do enter $L_2$. The FL anions were pulled into the intersection, close to $L_2$, by the electric field, and then a portion of the FL anions are pushed from the intersection into $L_2$ by the pressure-driven flow ($V_{B}$) into this channel while the remainder of the FL anions go to SW via $L_3$. It is noted that the application of the potentials generates a curved electrical field around the corner of the intersection, which can be visualized from the movement of the FL anions in the intersection. Thus, the SBP step serves as a means of introducing low-conductivity buffer into a portion of $L_4$ to aid stacking during the FASI step, which is discussed below.

**Field-Amplified Stacking Injection.** During the FASI process, a voltage $U_0$ is applied at BW while SW is grounded, SR is floated, and a voltage, $U_1$ (0.26 kV), is applied at BR, as indicated in Figure 1c. The FASI process in many respects will be just the reverse of the SBP injection step, since $L_4$ and $L_3$ have the same dimensions and assuming that the buffer in $L_3$ approximates that in $L_1$ by the time the FASI step is initiated. The application of the reversed-polarity voltage between BW and SW creates an EOF moving between $L_4$ and $L_3$; however, the bulk flow in $L_3$ ($V_3$) will be significantly larger than the bulk flow in $L_4$ ($V_4$). Thus, the bulk flow in $L_3$ will induce a siphoning (pulling) effect in the intersection and will cause siphoning flows in $L_1$ ($V_1$), $L_2$ ($V_2$), and $L_4$ ($V_4$) in a manner similar to the pressure-induced flows in
the channels during the SBP injection. In addition, the small voltage applied to BR will also generate an electric field strength in L2 that is in the direction of BR at the beginning of the FASI step and reverses as the FASI proceeds. The magnitude of the bulk velocity in channel L3 during FASI will be similar to what was obtained in channel L1 during the SBP injection (eqs 3a and 5a). The electrophoretic velocity of FL anions (V_{epFL}) is in the opposite direction of the bulk flows in L3 and L4. For the model system discussed in the SBP injection process, the apparent velocity of FL anions in channel L3 during the FASI process is in the direction of the SW reservoir (V_{3x} > V_{epFL}), i.e., the bulk flow in channel L3 carries FL anions into the SW reservoir without being injected into the separation channel L4 for stacking. However, the electrical field distribution during the FASI processes, similar to the SBP injection, produces curved potential lines in the intersection due to the turns of the channels. The FL anions moving into the intersection from SR during the FASI process are pulled into the separation channel by the electrical field and are electrophoretically drawn toward the BW reservoir and end up stacking at the interface between the low- and high-conductivity buffers in L4. Simultaneously, the low-conductivity sample buffer introduced during the SBP injection is pushed out of L4 and into L3 by the bulk flow in L4. Thus, the FL anions driven into L4 from SR are stacked in L4 at the discontinuous buffer interface, and the buffer interface is simultaneously swept back toward the intersection.

The FASI process was visualized by using a neutral (BODIPY 505/515) and an anionic (FL) dye. As a result of the SBP injection, the neutral dye is essentially uniformly distributed in the intersection and channels that are in the field of view at the initiation of the FASI process (Figure 4a). However, as time progresses, the neutral dye can be seen to clear from L4 as a result of the induced siphoning flow from L1 and the corresponding siphoning flow in L2. In L4, a bright zone of stacked FL anions is visible near the intersection at the end of the FASI process (Figure 4b). The stacked FL anions in L4 originate from a continuous flow of FL anions being pulled from SR into the intersection by a combination of the siphoning flow induced in L1 and the curved electric field existing in the intersection. The induced flow from L3 continues to provide FL anions for injection until the dispensing voltage setup is switched on. The injected amount of FL anions is related to the volume of sample solution from channel L1 flowing into the intersection. Thus, the rate of introduction of FL anions into the intersection is controlled by the siphoning flow in L1, which can be controlled by adjusting the relative length of each channel. Significantly, a higher concentration enhancement will be achieved by reducing the length of L1.

Optimization of SBP Injection Length. The SBP length was found to be crucial for the final detection enhancement obtained in the overall process. The optimum SBP length was determined by varying the combination of the SBP injection time (Figure 1b) and the FASI duration (Figure 1c) while holding the other steps (Figure 1a and d) constant and monitoring the resulting signal for FTTC and FL (Figure 5). It can be seen that if the SBP injection time was too short or too long (i.e., if the SBP was too small or too long), a somewhat lower peak signal was obtained for both FITC and FL. If the SBP injection is too short, the amount of time available for stacking analyte anions from the siphoning-induced flow from L1 is reduced, and correspondingly, the signal enhancement is reduced. If the SBP plug is too long, several effects will combine to reduce the signal enhancement. Under ideal conditions with no diffusion and convection between buffers, longer SBP (L1)
FASI processes for FL anions were examined in L\textsubscript{4} at several
monitored in L\textsubscript{4} at 50 μm was found to be optimal.

These experiments, a SBP injection time of 15 s
mixing of the discontinuous buffer system, leading to peak
broadening. In addition, a longer SBP plug needs a longer SBP
injection time, which will cause more significant diffusion and
mixing of the discontinuous buffer system, leading to peak
broadening. In these experiments, a SBP injection time of 15 s
was found to be optimal.

Process Monitoring. In Figure 6, the SBP injection and the
FASI processes for FL anions were examined in L\textsubscript{4} at several
locations displaced from the intersection. In Figure 6a, the
monitoring was done at 50 μm from the intersection using a 30-s
loading period (Figure 1a), followed by a 15-s SBP injection
(Figure 1b) and an extended FASI period (60 s, Figure 1c) to
allow the visualization of the stacked peak’s moving into the
intersection. It can be seen that a small amount of analyte was
introduced into L\textsubscript{4} during the loading process, but none was
introduced during the 15-s SBP injection time (30–45 s in Figure
6a), whereas at the beginning of the FASI process (45–52 s in
Figure 6a), FL anions are injected into L\textsubscript{4} (as shown in Figure
4b), and finally, the stacked FL anions move past the detection
point, as indicated by the large peak observed from 50 to 70 s in
Figure 6a. The FASI process and the dispensing step were
monitored at different locations along the separation channel with
the conditions given in Figure 1. Peaks 1 and 2 are the stacked
analytes during FASI and dispensing processes, respectively.

should not adversely affect the signal because the amount of
analyte anions injected into L\textsubscript{4} is a result of the siphoning flow
induced in L\textsubscript{4}. However, the siphoning flow from L\textsubscript{2} contains high-
conductivity running buffer, which will mix with the low-
conductivity sample buffer already present in channel L\textsubscript{3}. The
increased conductivity of the buffer in L\textsubscript{4} reduces the bulk flow in
L\textsubscript{3} and correspondingly reduces the siphoning flow from L\textsubscript{4}
and results in a decreased number of anions being injected into L\textsubscript{4}
for stacking. In addition, a longer SBP plug needs a longer SBP
injection time, which will cause more significant diffusion and
mixing of the discontinuous buffer system, leading to peak
broadening. In these experiments, a SBP injection time of 15 s
was found to be optimal.

Conductivity Ratio Effect. Sample stacking is based on the
uneven distribution of the electric field in a discontinuous buffer
system, leading to the rapid movement of charged species in the
low-conductivity matrix, with immediate slowing down at the
boundary of the two buffers. Theoretically, the concentration
enhancement should be proportional to the conductivity ratio of
the two buffers, and therefore, the highest possible ratio should
be used to provide maximal signal enhancement. However, due
to pressure-driven laminar flow and diffusion/convection mixing
of the discontinuous buffers, the interface and the conductivity
ratio of the two buffers are rarely kept ideal in practical operations;
thus, the concentration enhancement generally is lower than
expected. A conductivity ratio of 10 has been reported to produce
optimal enhancement and to maintain resolution in normal
stacking mode. The effect of the conductivity ratio of the
running buffer to SBP was investigated by monitoring the
fluorescence response for FL anions at 50 μm from the intersection
in the separation channel (Figure 7) using the SBP/FASI voltage
program given in Figure 1. When the SBP had the same
conductivity as that of the running buffer (γ = 1), no sample
stacking was obtained. The signal enhancement increased as the
conductivity ratio increased, with the largest enhancement
occurring with a conductivity ratio of 70, obtained using 0.50 mM
tetraborate as the SBP and 35 mM tetraborate as the running
buffer. The SBP/FASI process appears to tolerate a larger
conductivity ratio than the previously reported factor of 10, and
this may be due to the fact that the low conductivity buffer is
pushed out of the separation channel during the FASI step.

Detection Enhancement. The optimized SBP injection and
FASI process was compared to the standard pinched injection
(Figure 1a and d only) to examine the achievable detection
enhancement. For the SBP/FASI process, the sample solution of
FITC, FL, and 5-FAM at 50 nM each was prepared in 0.50 mM
tetraborate buffer (pH 8.3), whereas for the standard pinched
injection process, FITC, FL, or 5-FAM at 400 nM each were
dissolved in 35.0 mM tetraborate, pH 8.9. The detection
enhancements were 94-, 108-, and 160-fold for FITC, FL, and 5-FAM,
respectively. Their individual limits of detection, defined at 3× the signal-to-noise, are lowered by 100-, 110-, and 170-fold. The enhancements for FITC, FL, and 5-FAM are different and follow their electrophoretic mobilities, with 5-FAM giving the largest and FITC giving the smallest enhancements. Thus, it can be concluded that the enhancement is more advantageous for species with high electrophoretic mobilities in this stacking injection scheme.

**Calibration Curves.** FL and 5-FAM were used to evaluate the concentration linearity of the SBP injection/FASI process. The concentration ranges for the standards were from 1 to 64 nM for FL and from 2 to 60 nM for 5-FAM, while the concentration of FITC was kept constant at 15 nM to serve as the internal standard. Both the peak height and peak area ratios demonstrated good linearity for both analytes over the concentration ranges investigated. The equations and the correlation coefficients for the peak height ratios are $y = 0.151x + 0.08$, $R^2 = 0.998$ and $y = 0.073x + 0.026$, $R^2 = 0.999$ for FL and 5-FAM, respectively. The equations and the correlation coefficients for the peak area ratios are $y = 0.175x + 0.071$, $R^2 = 0.999$ and $y = 0.126x + 0.073$, $R^2 = 0.999$ for FL, and 5-FAM, respectively, where $x$ is the concentration axis and $y$ is the response axis. The percent RSDs of peak height and peak area ratios relative to FITC were each less than 4% for both FL and 5-FAM. Additionally, the percent RSDs ($n = 4$) of the uncorrected migration times, peak heights and peak areas for FL and 5-FAM are acceptable (below 5%)

**CONCLUSIONS**

The flows and analytic movements in μCE are complicated processes due to the open communication between the various channels, especially when involving a discontinuous buffer system. The valveless branched channels need to be taken into account when a discontinuous buffer system is involved, and the pressure-driven/induced pumping effect in the channel intersection can be utilized to achieve liquid transport and ion discrimination. The theoretical and experimental investigations demonstrated that FASI is a robust and useful online preconcentration method on commercially available microchips. This scheme produced around 100-fold detection enhancements for the analytes used, but the detection enhancement can be further improved by employing different chip designs. It should be noted that the current preconcentration scheme works only for anionic species, but use of a cation-modified surface would allow for the determination of cationic analytes. Therefore, this FASI scheme may provide some clues for the development of on-line sample preconcentration in μCE and will be of particular significance to the detection capability in emerging miniaturized analytical instrumentation.

**ACKNOWLEDGMENT**

Thanks to Dr. James J. Bao (School of Pharmaceutical Science and Technology, Tianjin University, China) for his review of the manuscript. Financial support was provided by the NIH (GM 69547) and the University of Cincinnati.