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Articles

One-Step Concentration of Analytes Based on Dynamic Change in pH in Capillary Zone Electrophoresis

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A novel strategy for one-step concentration of analytes during capillary electrophoresis (CE) is presented. A short platinum wire was inserted into the 75-μm-i.d. separation capillary. When a high voltage was applied for CE separation, a sharp pH gradient along the capillary was created dynamically by the electrolysis of water in the running buffer. Concentration of a large volume of injected analytes was accomplished by the change in analyte charge due to the dynamic pH gradient. Depending on the polarity of the applied potential and the direction of electroosmotic flow, either anions or cations can be concentrated. Several hundredfold concentration factors were achieved. Fluorescence imaging by a CCD camera was used to monitor 10 cm of the capillary near the platinum wire during the concentration process. The observations are consistent with a sweeping mechanism.

On-line preconcentration has been widely employed in capillary electrophoresis (CE) for improvements of concentration detection limits and separation efficiencies.[1−10] Preconcentration of analytes can be carried out by injecting large amounts of sample and subsequently narrowing the sample zone prior to separation. Many sample-stacking methods have been developed by manipulating these two operations. Field-enhanced sample stacking (FESS)[1−6] is based on the conductivity difference between the sample zone and the running buffer to effect preconcentration. The low conductive sample matrix will experience a higher electric field strength, and analyte ions will move faster than the ions of the background electrolyte (BGE). Thus, a larger amount of analyte ions can be injected into the capillary without compromising the separation efficiency. Stacking takes place at the boundary between the sample zone and the BGE. Up to 1000-fold preconcentration factors have been reported.[4] Large-volume sample stacking (LVSS)[7−10] is another approach for sample preconcentration. To obtain a narrow sample zone after a large volume of analyte is injected into the capillary, many techniques have been reported. Hjertén et al.[11,12] proposed a variety of strategies for sharpening the sample zone, including isoelectric focusing in a pH gradient, zone electrophoresis toward a small-pore gel, a gradient in effective cross-sectional area (to create a gradient in electric field), a gradient in conductivity or viscosity, and a combination of displacement electrophoresis and a counterflow. For peptides and proteins, a 1000-fold stacking factor was obtained.[12] However, sample focusing and mobilization are performed in two separate steps, making the approach inconvenient to implement. Quirino and Terabe[13,14] introduced the sweeping concept for sample stacking in micellar electrokinetic chromatography (MEKC). This technique is based on micelles that can pick up and accumulate analyte molecules when they penetrate the sample zone during separation. The sample zone is narrowed by a factor of $1 + k$ (k being the retention factor). Thus, it is suitable for strongly retained analytes. Palmer[15−17] developed a technique for stacking from high-salt matrixes by combining sample sweeping and micelle stacking. Chen et al.[18,19] used a dynamic pH junction or complexing agent for preconcentration of weakly acidic compounds and nucleotides by introducing two different buffers in the sample and the background electrolyte zones.

In the FESS, LVSS, or sweeping approaches, discontinuous buffers were always used to provide different conductivity, pH, viscosity, or micelles between the sample zone and the running buffer so that stacking occurs at the boundary of the two different zones. However, sometimes these discontinuous buffers may

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deteriorate the separation performance. Discontinuous buffers are also difficult to create reproducibly. Another step is usually needed to complete the stacking process, such as removing the water plug for LVSS.[8,10]

In this work, we demonstrate a unique sample concentration technique in a continuous buffer system in CE. A Pt wire is inserted into the fused-silica separation capillary. Electrolysis of water at the Pt wire inside the capillary on application of voltage can build up a sharp dynamic pH gradient, which instantaneously changes the local electroosmotic flow (EOF) but more importantly the charge on the analyte. The sample zone narrows because different migration velocities are associated with the varying charges on the analyte under the dynamic pH gradient. Thus, sample concentration can be achieved efficiently and in one operation.

EXPERIMENTAL SECTION

Chemicals. Poly(vinylpyrrolidone) (PVP) with MW 360 000, histidine, and tris(hydroxymethyl)aminomethane (Tris) were of analytical reagent grade from Sigma (St. Louis, MO). Didodecyldimethylammonium bromide (DDAB) was obtained from Fluka. Fluorescein, 5(6)-carboxyfluorescein, 2,7-diacetate dichlorofluorescein (2,7-dAcCl-fluorescein), fluorescein isothiocyanate (FITC), coumarin 334, rhodamine B, and rhodamine 6G were all analytical grade. The sample solutions were prepared by dissolving the analytes in the running buffer to eliminate the effects of field-amplified preconcentration.[2]

Capillary Electrophoresis. The home-built CE setup with laser-induced fluorescence (LIF) detection was described in our previous work.[20] Briefly, 5 mW of a 488-nm laser light line from an Ar^+ laser (model Innova 90, Coherent, Palo Alto, CA) was used for excitation. The laser beam was focused onto the 75-μm-i.d. fused-silica capillary by a 1-cm focal length convex lens. A 20× objective lens was used to collect the fluorescence perpendicular to the laser beam. A 530-nm long-pass filter was placed before the photomultiplier tube (PMT) to eliminate laser scattering. The fluorescence signal from the PMT was converted by a 10-kΩ resistor, fed into a 24-bit A/D converter (Lawson Labs, Kalispell, MT), and stored in a desktop computer at 2 Hz.

A 60-cm-long (40 cm effective), 75-μm-i.d. bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ) was used for CE separation. Short platinum wires (Goodfellow, Berwyn, PA; 25−50-μm o.d., 2−3 cm long) were carefully inserted into the capillary. The capillary and Pt wire were then rinsed with 1 M HCl for 5 min, 10% HNO_3 for another 5 min, and finally running buffer for 10 min. Long sample plugs were injected hydrodynamically before applying high voltage. Normally, the Pt wire stays in a fixed location once it is inserted.

Fluorescence Imaging. A charge-coupled device (CCD) camera (Photometrics, Tucson, AZ) with a 28-mm Nikon camera lens was used for image capture. The optical setup for CCD imaging was similar to the one we reported earlier.[21] The polyimide coating of ~10 cm of the capillary near the Pt wire was burned off to make the capillary optically transparent. The 488-nm Ar^+ laser was expanded horizontally to ~15 cm upon the detection window by a 5-cm focal length cylindrical lens. Another 5-cm focal length cylindrical lens was used to focus the laser beam vertically onto the capillary. The CCD was arranged in a way such that ~350 pixels were used to view the 10-cm detection region.

RESULTS AND DISCUSSION

Creating a Sharp pH Gradient. pH gradients have been used in capillary isoelectric focusing by introducing ampholytes into the capillary.[22,23] This type of pH gradient is continuous and can only be used for focusing analytes with an isoelectric point. Another approach is the discontinuous buffer systems employed by Hjertén et al.[11,12] or Chen et al.[18,19] However, the sharpness of the pH gradient is restricted by diffusion and by the buffer pH. Electrolysis of water at the inlet and outlet vials in CE is a common phenomenon and has been observed by many researchers.[24−27] Sweedler et al.[25] had confirmed that the buffer pH was quickly altered when only a small amount of buffer solution is present in the reservoirs. For example, for 20 mM phosphate, the buffer pH changed from 7.2 to >13 in several seconds.

Here, when a Pt wire is inserted into the separation capillary, the electrochemical reactions are a bit different. When voltage is applied, the wire floats at a certain voltage depending on its location along the capillary. One end of the Pt wire therefore functions as an anode while the other end functions as a cathode. OH^- and H^+ are generated at the corresponding ends and move toward the anode and cathode, respectively. While OH^- or H^+ are continuously generated at the Pt wire, a pH gradient along the capillary is created as these ions begin to titrate the buffer ions and propagate down the column, as shown in Figure 1. The length of the Pt wire is not important in this scheme. The pH gradient created under this condition is very sharp because the concentration of H^+ or OH^- is extremely high in the nanoliter volumes near the wire. This sharp pH gradient is a dynamic one. According to Faraday's equation, the amount of H^+ or OH^- produced per unit time is proportional to the separation current. So the gradient in the capillary is fairly easy to control by adjusting the applied voltage and the concentration and the conductivity of the BGE.

However, to make this approach practical for CE, we first need to suppress bubble formation by the electrochemical reactions. Since the amount of bubbles is dependent on the separation current, a buffer with low conductivity is preferred. Also, a low ionic strength buffer is preferred to ensure a sharp pH gradient (fewer buffer ions per unit length to titrate). So, histidine was chosen as the running buffer due to its low conductivity and good buffer capacity.[28] We found no noticeable bubble formation during the separation when the histidine concentration was less than 30 mM. Therefore, a 15 mM histidine solution at pH 7.0 was used throughout this work. To work with high ionic strength buffers, the capillary will have to be cooled to avoid bubble formation.

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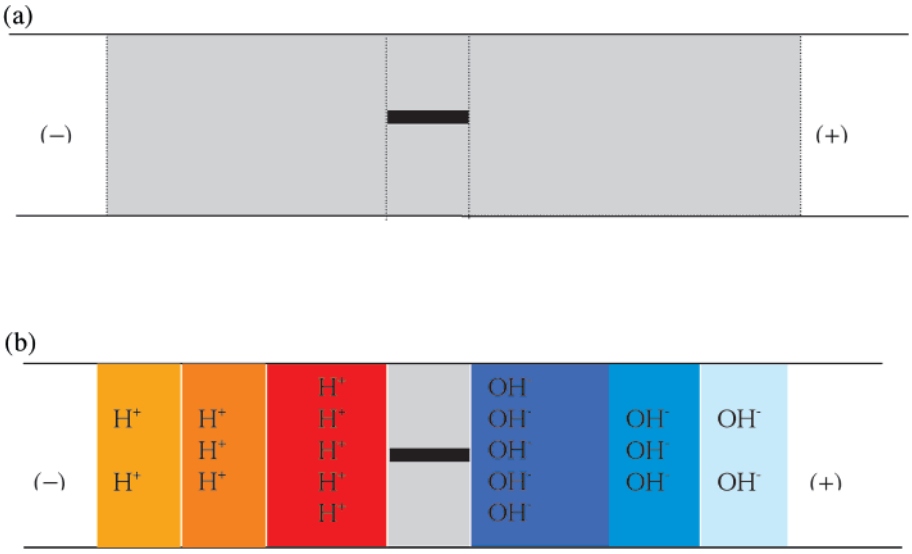

Figure 1. Schematic illustration of the creation of a sharp pH gradient by electrolysis of water when a Pt wire is inserted into the capillary: (a) before voltage is applied; (b) after voltage is applied.

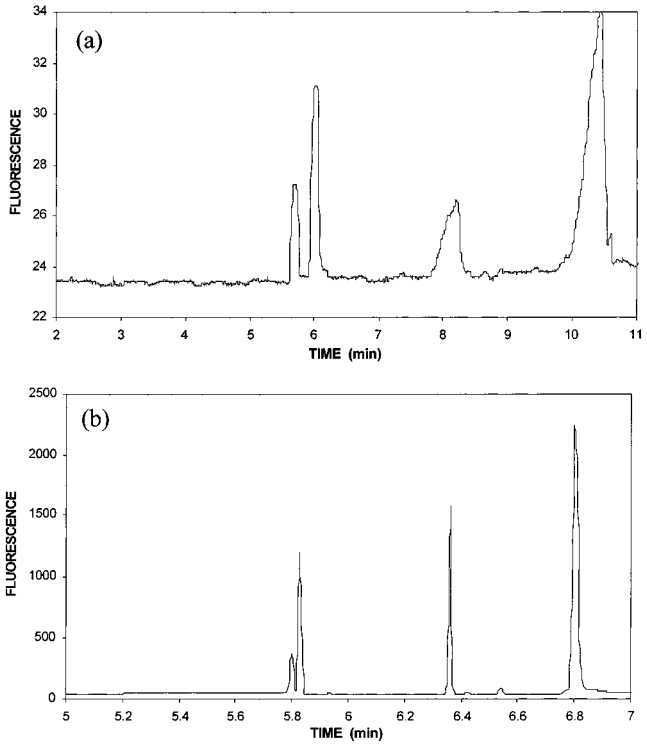

Figure 2. Electropherograms of anionic analytes. In the order of elution, these are 5(6)-carboxyfluorescein (2.8×10^{-9} M), fluorescein (1.3×10^{-9} M), and 2,7-diacetate dichlorofluorescein (2.6×10^{-9} M). (a) A 10-s injection in a normal capillary and (b) 3-min injection with a Pt wire inside the capillary. The BGE is 15 mM histidine (pH 7.01) in 0.2% PVP buffer. All samples were dissolved in BGE buffer. Capillary total length, 60 cm; effective length, 40 cm; Pt wire, 2 cm with 25-μm o.d. at the inlet end; and voltage applied, −20 kV.

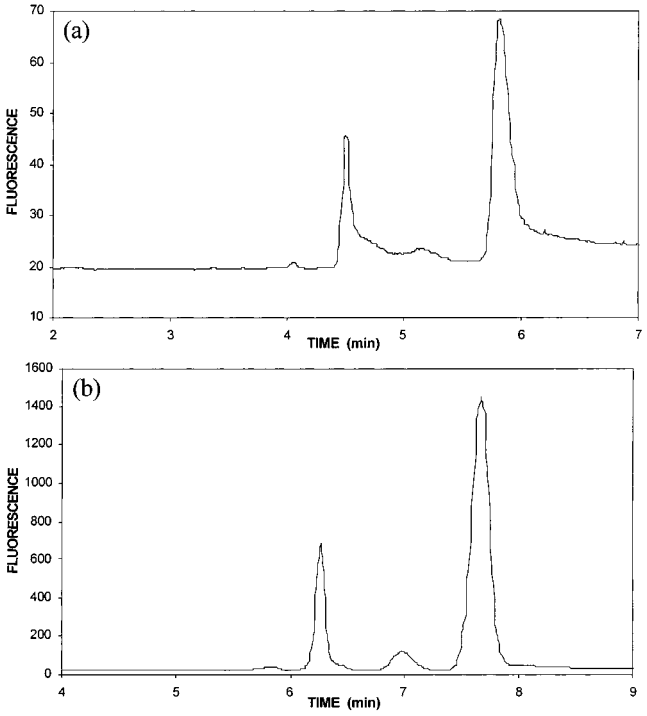

Figure 3. Electropherograms of cationic analytes. In the order of elution, these are rhodamine 6G (1.04×10^{-9} M) and rhodamine B (2.48×10^{-9} M). (a) A 10-s injection in a normal capillary and (b) 1-min injection with a Pt wire inside the capillary. BGE is 15 mM histidine (pH 7.01) in 0.01% DDAB buffer. All samples were dissolved in BGE buffer. Capillary total length, 60 cm; effective length, 40 cm; Pt wire, 2 cm with 25-μm o.d. at the inlet end; and voltage applied, −9 kV.

Concentration of Ionic Analytes. Several fluorescein derivatives were selected as negatively charged model analytes.[29] Rhodamine B and rhodamine 6G were used as positively charged model analytes. Then 0.2% PVP was added to the buffer to suppress EOF.[30]

Figure 2 shows the electropherograms of four anionic fluoresceins obtained without and with a Pt wire inserted into the capillary. For normal CE, a 10-s hydrodynamic injection time with 20-cm height differential at the inlet end was used. Under concentration conditions, the injection time was 3 min. Concentra-

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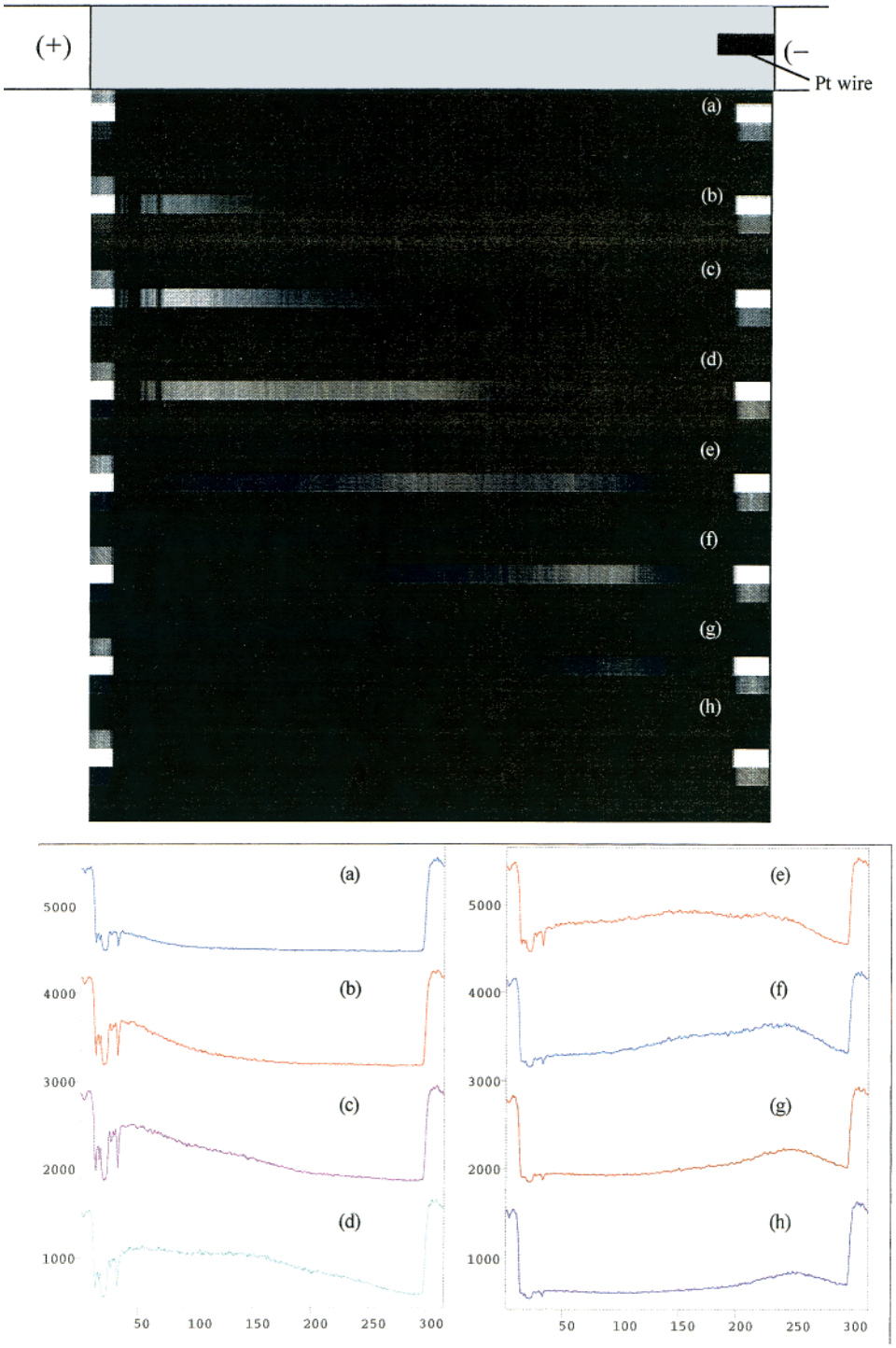

Figure 4. Images of a short sample plug of coumarin 334, a neutral marker. Capillary total length is 70 cm with a 10-cm observation window. BGE, 15 mM histidine (pH 7.01) with 0.2% PVP; Pt wire, 3 cm with 50-μm o.d. at the inlet end; and voltage applied, −7 kV.

tion and separation were thus implemented in one step. For cationic analytes, the polarity of the applied voltage and the position of the Pt wire were kept exactly the same as for the anionic analytes. However, DDAB was added in the running buffer to obtain reversed EOF.[31] Electropherograms of the cationic analytes are shown in Figure 3. By comparing the peak heights of the individual analytes, more than several hundredfold concentration factors for anionic analytes were observed. A 30-fold concentration factor for cationic analytes was found. The ratios of peak areas in the two sets of electropherograms are consistent with the ratios of the injected volumes (~18× in Figure 2 and ~6× in Figure 3 for all peaks). In addition, the concentrated zones are substantially sharper for both Figures 2b and 3b, thus producing further increase in the peak heights.

On-Capillary Imaging. To elucidate the stacking mechanism, an imaging system was set up for monitoring the whole process.

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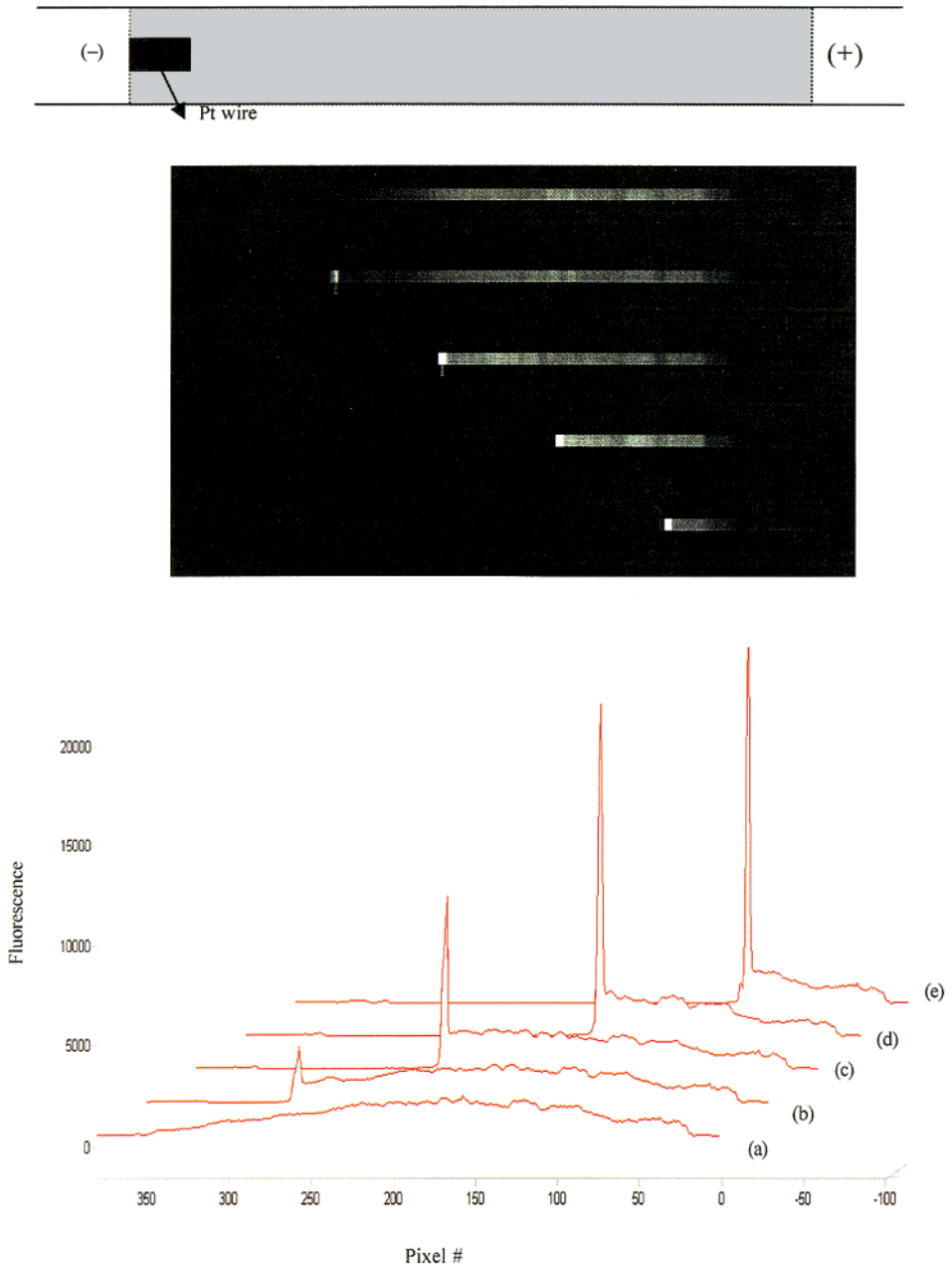

Figure 5. Images of a large-volume sample sweeping process. The fluorescein sample was filled into the whole capillary prior to the application of voltage. Other conditions are as stated in Figure 4.

In the first experiment, a ~4-cm-long coumarin 334 (a neutral molecule) plug was injected into the capillary hydrodynamically from the left side and pushed to the observation window just short of the field of view, as shown in Figure 4a. After applying voltage, the plug moved toward the cathode due to electroosmotic flow (Figure 4b−h). The sample plug retains its original intensity distribution and its length throughout, as depicted in the individual electropherograms.

In another experiment, we filled the entire capillary with 10^{-6} M 5(6)-carboxyfluorescein to simulate large-volume sample concentration (Figure 5). The low fluorescence intensity corresponds to the initial concentration of the analyte. After applying voltage, the sample zone near the Pt wire became brighter first, while the intensity of sample zone far away from the Pt wire did not change. The sharp bright zone continued to move toward the anode at a higher speed than the rest of the zone and became brighter and brighter. Also, the signal intensity along the capillary dramatically decreased after the bright sample zone passed through that region. This is indicative of a sweeping mechanism. We can clearly see that concentration takes place only at the boundary of the sample zone.

EOF Behavior. From our previous work,[29,30] PVP can effectively suppress EOF when the buffer pH is less than ~9. For example, in the normal CE mode (Figure 2a), several anionic analytes can be eluted out by applying a negative voltage in 0.2% PVP buffer.[29] To examine the effect of electrolysis of water on EOF, a neutral marker, coumarin 334, was used to measure the EOF. The EOF with a Pt wire inside the capillary in 15 mM histidine buffer (pH 7.0) containing 0.2% PVP was ~2.04×10^{-4} $cm^2\ V^{-1}\ s^{-1}$, which is 10 times higher than that in 0.1% PVP in 1×

TBE buffer (pH 8.2) in normal bare fused-silica capillaries.[31] The EOF in the presence of a Pt wire was very strong because of the low ionic strength despite the low pH and high PVP concentration. As voltage is applied, strong EOF initializes from the small region near the cathode end of the Pt wire because, at high pH, PVP cannot protect the silanol groups from ionizing.[30] As time goes on, the OH^- would titrate the buffer ions and migrate upstream due to its high electrophoretic mobility. Because more and more OH^- is generated with time, EOF increases further. On the other hand, the bulk liquid is driven by EOF toward the Pt wire. The buffer solution near the Pt wire, which has been depleted by the moving OH^-, is then refreshed by the EOF. So the net EOF reaches a constant high value by the balance of fresh OH^- and new buffer solution near the Pt wire.

This change in EOF does not directly participate in the sample concentration process. The motion of an analyte ion is always governed by the balance between its electrophoretic driving force and the electroosmotic flow. If the entire injected plug has the same electrophoretic velocity, the plug length and the local concentrations do not change with the change in EOF. This is illustrated in Figure 4 for the neutral marker coumarin 334. No zone sharpening and no change of direction can be observed. The dynamic change in EOF, however, affects the net migration time when a Pt wire is present. Figure 3b shows that the reversed EOF is gradually being reduced, and migration times become longer than those in Figure 3a.

Mechanism of Sample Sweeping. According to the experimental results above, a sweeping mechanism can be proposed. First, OH^- is produced at the cathodic end of the Pt wire by electrochemical reaction; thus, a sharp pH gradient was created along the capillary by the migration of H^+ and OH^-, as shown in Figure 1b. The charge of the analyte molecule is affected by the pH gradient. For these anionic analytes, the electrophoretic mobilities dominate the EOF. So, they would migrate out from the anode end in the normal CE. When a Pt wire is inserted, almost instantaneously, a pH gradient is formed. OH^- ions migrate faster then the analyte ions (against EOF). When the analyte molecules meet the increased pH region, they will ionize further since fluoresceins have several phenolic groups. The migration velocity of the molecules at the proximal part of the sample zone became faster compared with those at the distal part due to the increased electrophoretic mobility. Thus the sample zone was compressed, as shown in Figure 5. The higher-charged fluorescein molecules therefore keep sweeping up the unconcentrated analytes in the initial sample plug (Figure 5). A simple view is that the dynamically generated OH^- sweeps all analyte molecules into a very narrow zone and moves these toward the detection window. Therefore, a large concentration factor was obtained. It should be noted that separation of the analyte ions starts as soon as voltage is applied. Without the Pt wire, the long injection plugs would have led to step increases in fluorescence intensity at the detector. The sweeping effect compacts each (separating) analyte zone into sharp peaks to produce a high separation efficiency by the time the zones reach the detector. It is interesting to note that the separation depends on the charge of the analyte ions both before and after they are concentrated.

For cationic analyte, reversed EOF was needed to drive the analyte toward the CE anode (detector end) with or without the

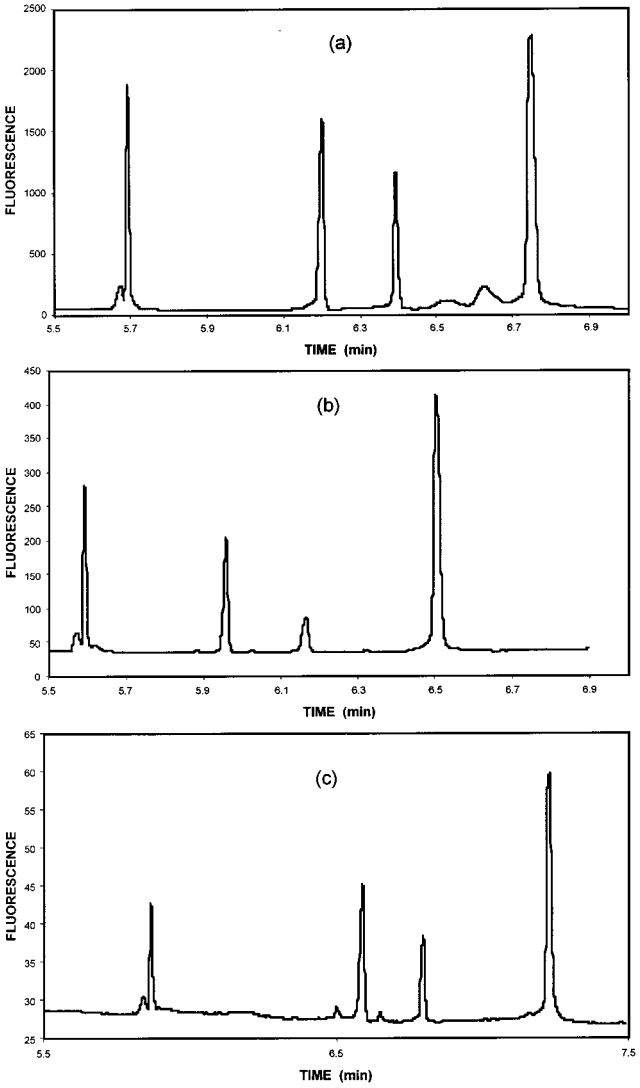

Figure 6. Electropherograms of sweeping at different initial concentrations of anionic analytes. In the order of elution, these are 5(6)-carboxyfluorescein, fluorescein, FITC, and 2,7-diacetate dichlorofluorescein: (a) 10^{-9}, (b) 10^{-10}, and (c) 10^{-11} M. Other conditions are as stated in Figure 2.

Pt wire. Again, the directions of the EOF and the electrophoretic mobility were opposite to each other. The analyte molecules proximal to the Pt wire (high pH) would no longer oppose the reversed EOF after neutralization of the positive charges at the high pH front. These molecules would catch up with the trailing part of the sample zone. Similarly, the sample zone became narrower and concentration took place. The zones were separated from one another based on the differences in electrophoretic mobilities before they were neutralized by the moving front. That is, sweeping occurs during and after separation. However, unlike the case of anionic analytes, the concentration factors are not as large. This is because, for this combination of electrophoretic and electroosmotic velocities, part of the long injected sample zone actually passes the detector before sweeping is complete. That is, the pH front is not fast enough to effectively concentrate the entire moving sample plug. Concentration factors should be further improved if the magnitude of reversed EOF is carefully controlled relative to the electrophoretic mobilities or if longer capillaries are used.

Reproducibility. Mixtures of five 10^{-9} M fluorescein derivatives were repeatedly injected to test the reproducibility of this preconcentration method. More than 20 runs showed a very similar (±10% in peak heights) sweeping effect. Fluorescein mixtures with different concentrations were also used to test the concentration efficiency. As shown in Figure 6, a reasonably linear relationship between the injected concentration and peak height was achieved. However, the slope of the working curve (= 0.7) is not equal to unity. This can be explained by zone-broadening effects due to mismatch in the electrophoretic mobilities of the BGE and the analyte as governed by the Kohlrausch function.[32,33] The same effect also explains why not all peaks show the same concentration factor (increase in height) even though the peak areas are always predictable from the hydrodynamically injected volumes.

CONCLUSIONS

A one-step sample concentration method for CE was developed by inserting a short platinum wire into the separation capillary. Neither changing of the buffer vials nor switching of the polarity of the applied potential was necessary. Sample sweeping was achieved by instantaneously changing analyte charge by a sharp OH^- gradient. This mechanism was confirmed by in situ monitoring of the electrophoresis process. This new method can be used for any ion provided that its charge changes with pH. That would include any weak acid or weak base. The key requirement is that the dynamic pH front propagates down the column at a higher speed than that of the analyte ion. In principle, such sweeping can be implemented by using small-volume buffer reservoirs to magnify the effect of pH change due to electrophoresis. In practice, the Pt wire produces a sharper gradient faster because of the small local volume inside the capillary tube. The physical length of the Pt wire is not important. Within that region, the electric field gradient is zero. However, EOF will drive all analytes in that zone and those before that zone back out the injection end of the capillary. So, the length of the Pt wire does not contribute to band broadening, as is obvious in Figures 2 and 5. The placement of the Pt wire from the front of the injection zone, on the other hand, determines the length of the injection plug that will be concentrated. Over repeated usage, there does not appear to be any degradation in the Pt wire or the capillary.

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