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Sampling BIAS at Channel Junctions in Gated Flow Injection on Chips

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The commonly used gated injection scheme was examined and found to suffer from multiple levels of electrokinetic sampling bias, including a new type based on transradial electrokinetic selection (TREKS). TREKS occurs as analytes of differing electrophoretic mobilities migrate around the corner at a channel junction in a microchip. The overall sample bias in gated injection was shown to be time-dependent and resulted in a larger sample bias against components of negative electrophoretic mobility. A new injection procedure for microchip devices based on interstream diffusion at zero potential is proposed. Diffusion of molecules into the separation channel is the main driving force for this type of injection. The new scheme is shown to be useful for injection of complex samples with multiply charged components, such as peptide mixtures. This procedure allows sampling of volumes from 12 to 45 pL, reproducible retention times (RSD < 1.5%), and reproducible peak areas (RSD < 2.3%).

One of the challenges in fabricating a laboratory-on-a-chip is in the miniaturization of system components. Sample introduction, sample pretreatment, chemical reaction, separation systems, and detection flow cells must all be reduced in volume to handle 1−1000-pL samples.[1−4] An important question is how to move liquid to execute these operations in the chip. Reagent and mobile phase transport are generally achieved by electroosmosis in microfluidic systems. This form of transport is attractive, because it is simpler than pressure-driven flow. In addition, voltage-driven transport generates less band spreading. Finally, no moving parts are required.

There are, however, limitations with electrokinetic transport. One is that reagents and analytes are often transported at different rates. This leads to the well-known problem of sampling bias.[5] Electrokinetic sampling bias (*b*) is based on the fact that analytes differing in electrophoretic mobility enter microchannels from a sample reservoir at different rates. The relative difference in the rate at which two analytes enter a channel, that is, the sampling bias, is given by the equation

$$b = \frac{v_1}{v_2} = \frac{(\mu_{ep1} + \mu_{eo})}{(\mu_{ep2} + \mu_{eo})} \quad (1)$$

where v_1 and v_2 are the effective mobilities of analytes 1 and 2, respectively; μ_{ep1} and μ_{ep2} are their electrophoretic mobilities; and μ_{eo} is the electroosmotic mobility. This bias poses two problems in quantification. One is that an electropherogram or chromatogram will not reflect the true concentration of sample components. Another, more serious, problem is that analytes of negative electrophoretic mobility that electrophorese back against the electroosmotic flow will be present in a smaller amount for detection. As the term $(\mu_{epi} + \mu_{eo})$ becomes smaller, less analyte is entering the channel to be detected. Although this problem has been addressed with the hydrodynamic injection scheme in open-tubular CE systems, there is no equivalent solution on chips. Electrophoretic injection remains the only viable solution on chips.

Chemical and electrophoretic analyses on chips all suffer from electrokinetic sampling bias in electroosmotically driven flow, regardless of the injection mode. Sample aliquots are generally metered out for analysis at the junction of two channels. One channel leads from the sample reservoir to a waste well, and the other, from a buffer or reagent well to a waste well beyond the detection zone. Cross injection,[6] pinched-flow injection,[7,8] gated injection,[9−11] and variations of these techniques have all been explored on chips[12,13] and have been found to vary greatly in the concentration of components injected.[11] Cross injection suffers from the difficulty of controlling sample size, whereas pinched-flow injection solves this problem but allows limited variation in sample size.[7,8] Gated injection, in contrast, solves both of these

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problems and allows injection in the continuous flow mode as well.[12]

This paper examines sampling bias at the channel junction in gated flow injection systems on chips. It will be shown that certain types of injection protocols can aggravate the problem of electrophoretic sampling bias. Like the sampling bias already described in the literature, a new type of sampling bias will be described that discriminates against analytes of negative electrophoretic mobility. An alternative protocol is proposed to partially solve this sampling bias problem on chips.

EXPERIMENTAL SECTION

Reagents. Sylgard 184 PDMS base polymer and curing agent were purchased from Dow Corning (Midland, MI). 4-Styrenesulfonic acid, fluorescein isothiocyanate labeled bovine serum albumin (FITC−BSA), 2-acrylamido-2-methylpropanesulfonic acid (AMPS), methoxydimethyloctadecylsilane, rhodamine 110, and glycyltyrosine (Gly-Tyr) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium bicarbonate and potassium carbonate were purchased from Mallinckrodt (Paris, KY). Ammonium cerium(IV) nitrate was obtained from Lancaster (Pelham, NH). The peptide H-Gly-Phe-Glu-Lys-OH came from BACHEM Bioscience Inc. (King of Prussia, PA). Fluorescein-5-isothiocyante (FITC isomer I) was purchased from Molecular Probes (Eugene, OR).

Modification of PDMS Chips. PDMS separation columns were molded from a positive photoresist master as previously described.[14] A standard cross with 75-μm-wide, 10-μm-deep channels was used for visualization of flow profiles during injection procedures. A 3.9-cm-long COMOSS column with 5 × 5 μm particles and 3-μm-wide channels (Figure 1) was used for separation.[15] After oxidation, the molded PDMS column and cover slab were brought into contact to make an irreversible seal. The channels were modified with 4-styrenesulfonic acid or AMPS with ammonium cerium(IV) nitrate catalyst as described in the literature.[16] C_{18}−AMPS synthesis was also previously described.[16]

Instrumentation. Visualization of fluid movement in channels was achieved with a Nikon inverted eclipse TE-300 optical microscope via a TE-FM confocal-fluorescence system and fluorescent markers (10^{-5} − 10^{-4} M) as previously described.[17] Data collection was obtained with an epifluorescence system built in-house, as previously described.[18]

Injection of Samples. Peptide samples were labeled with FITC as described.[19] Rhodamine 110 and FITC−BSA were dissolved in 1 mM carbonate buffer to a concentration of 10^{-4}M. Electrokinetic injections were made using a gated injection procedure[10] involving a three-step protocol:

(i) Initially, flow in the chip was established by applying a 2500 V potential to the running buffer electrode (I) and 2000 V to the sample electrode (II) (Figure 1) while the electrodes in the waste reservoirs (III and IV) were grounded.

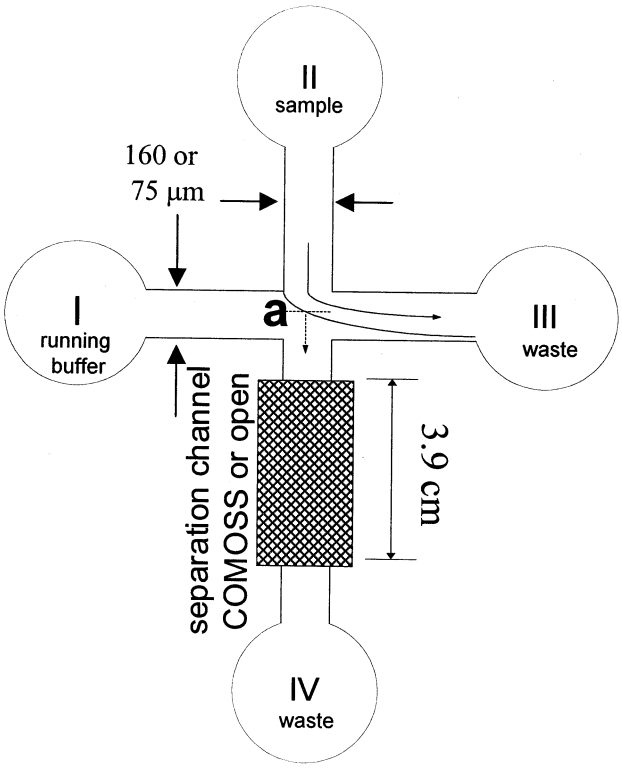

Figure 1. Schematic diagram of the channel junction used for sample introduction.

(ii) Gated flow injection was achieved by switching the potential on electrodes I and II to 0 and 1000 V, respectively, for a selected time. Larger samples were injected by increasing the time the system was left in this voltage pattern.

(iii) After injection, potentials were switched back to the initial values, 2500 and 2000 V on electrodes I and II, respectively.

This protocol allowed flow in the separation channel to continue uninterrupted.

The same procedure was used to achieve diffusion-based injections with one exception: the potential during the injection step (ii) was reduced to 0 on all of the electrodes for 2 to 20 s. Flow in all of the channels stopped when the electromotive force was interrupted, and analytes diffused into the channel junction. When voltage was reapplied, sample that diffused into the buffer stream going to the separation channel was carried forward for analysis.

RESULTS AND DISCUSSION

Sample Bias in Gated Injection. During the course of examining gated flow electrokinetic injection in chip based capillary electrochromatogaphy (CEC) systems, it was noted that sampling bias varied with the injection protocol. A sample consisting of an equimolar mixture of the FITC-labeled peptides FITC-Gly-Phe-Glu-Lys-OH, FITC-Gly-Phe-Glu-Lys(FITC)-OH, and FITC-Gly-Tyr-OH was separated using a polystyrenesulfonic acid stationary phase immobilized on collocated monolith support structures (COMOSS) fabricated in poly(dimethylsiloxane).[16] The elution profile in Figure 2a resulted from the normal mode of gated flow injection, that is, by electrokinetically driving sample into the separation channel (Figure 1) for 0.5 s at 1000 V potential. In contrast, the elution profile in Figure 2b was produced by dropping

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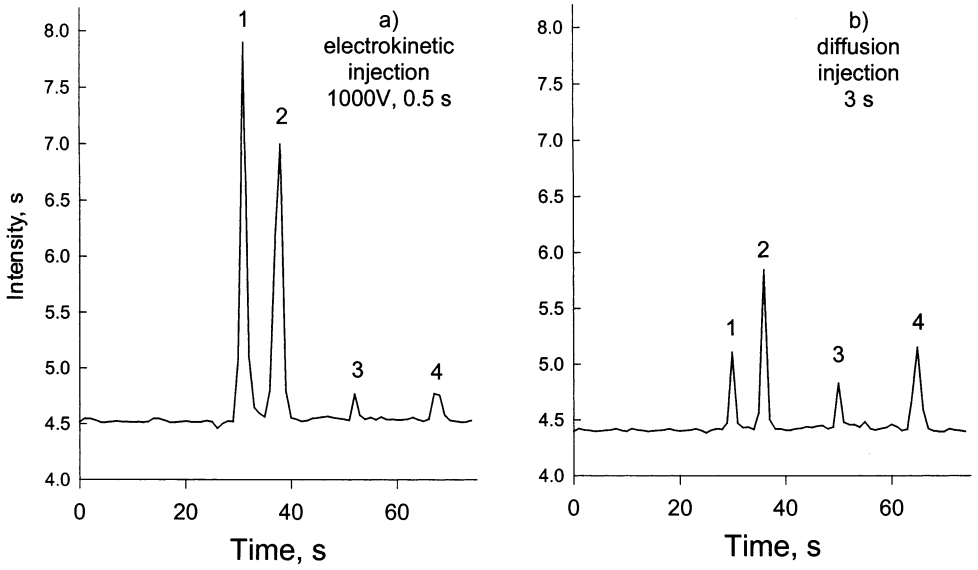

Figure 2. Separation of an FITC-labeled synthetic peptide mixture following electrokinetic (a) and diffusion-based (b) injection on a 4-styrenesulfonic acid modified microchip. Electrokinetic injection was achieved by applying 1000 V at electrode II for 0.5 s. Diffusion injection time was 3 s. Mobile phase, 1 mM carbonate buffer (pH = 9.0); separation potential, 300 V/cm. 1, FITC-Gly-Phe-Glu-Lys-OH; 2, FITC-Gly-Phe-Glu-Lys(FITC)-OH; 3, FITC; 4, FITC-Gly-Tyr-OH. Analyte concentrations: $1 + 2 = 4 = 5 \times 10^{-5}$ M.

the potential to 0 on all electrodes in the system for 3 s and allowing analytes to diffuse into the separation channel before reapplying potential to electrodes I and II. Clearly, there is discrimination against late eluting peaks in the gated flow injection mode. It is surprising that allowing analytes to diffuse passively into the separation channel as opposed to actively transporting them electrokinetically would make such a large difference. These results suggest that beyond the electrophoretic bias analytes experience as they are electrokinetically transported to the channel junction for injection, a second level of sampling bias occurs during the gated flow injection process.

Multiple Electrokinetic Selection. Gated flow injection is part of a multiple step electrokinetic process. As voltage is switched in step two of the gated flow injection protocol (see Experimental Section), sample is diverted into the separation channel. It will be assumed for the sake of discussion that when analytes move beyond point a, toward waste reservoir IV in Figure 1, they are committed irreversibly to the separation channel. In this case, individual electrophoretic mobilities will bias selection as analytes enter the vacant zone beyond point a. The amount (Q_i) of a particular analyte entering the zone beyond the point a in Figure 1 is expressed by the equation[20]

$$Q_i = \frac{(\mu_{epi} + \mu_{eo})tSVC_i}{L} \qquad (2)$$

where t is the time the sample stream has been diverted into the separation channel, V is the applied voltage, S is cross sectional area of the capillary, C_i is the concentration of analyte, and L is the length of the capillary. For example, if $C_1 = C_2$ in the sample reservoir, but their effective mobilities are in a ratio of 1:2, they will be biased to an amount of 1:2 during the injection.

Therefore, the effect of gated flow injection is that sample components will have experienced electrokinetic bias before reaching the detector. This bias is generally described by eq 1, which suggests that electrophoretic bias should be constant and independent of injection time. Yet it is seen in Figures 2 and 3 that the relative concentration of analytes varied with switching time. Some additional process must contribute to selection in the channel junction.

Spatial Distribution of Analytes at the Channel Junction. Attempts to observe this process were focused on visualization of analyte flow profiles in the channel junction using a confocal microscope fitted with a fluorescence detection system and a CCD camera. Rhodamine 110 and FITC−BSA, at a concentration 10 fold lower than the buffer, were chosen for these studies, because they varied substantially in both electrophoretic mobility and diffusion coefficient. Figure 4a shows the concentration profile of these model compounds as they move from the sample well through the channel junction to the waste well under identical conditions. It is clear that the smaller, faster moving rhodamine 110 traces out a larger turning radius than the slower moving, high-molecular-weight FITC−BSA. Overlaying these two profiles in Figure 4b shows that the turning radii of these two analytes differ by 20 μm in a 75 × 75-μm junction. A zone is formed beyond the turning radius of FITC−BSA that contains rhodamine 110 alone; i.e., a type of continuous moving boundary separation [21] occurs as the analytes turn the corner and move toward the waste reservoir. As with all moving boundary separations, the leading zone contains the analyte of highest electrophoretic mobility whereas trailing zones are composed of the leading analyte and progressively slower moving analytes.

A similar experiment was performed with the FITC labeled peptides FITC-Gly-Phe-Glu-Lys-OH (1) and FITC-Gly-Tyr-OH (4) that differed little in diffusion coefficient (Figure 4c). The observed difference in the channel junction turning radius of these analyes was 7 μm. Because of the diffusion coefficient similarity in this case, it was concluded that an electrokinetic phenomenon must

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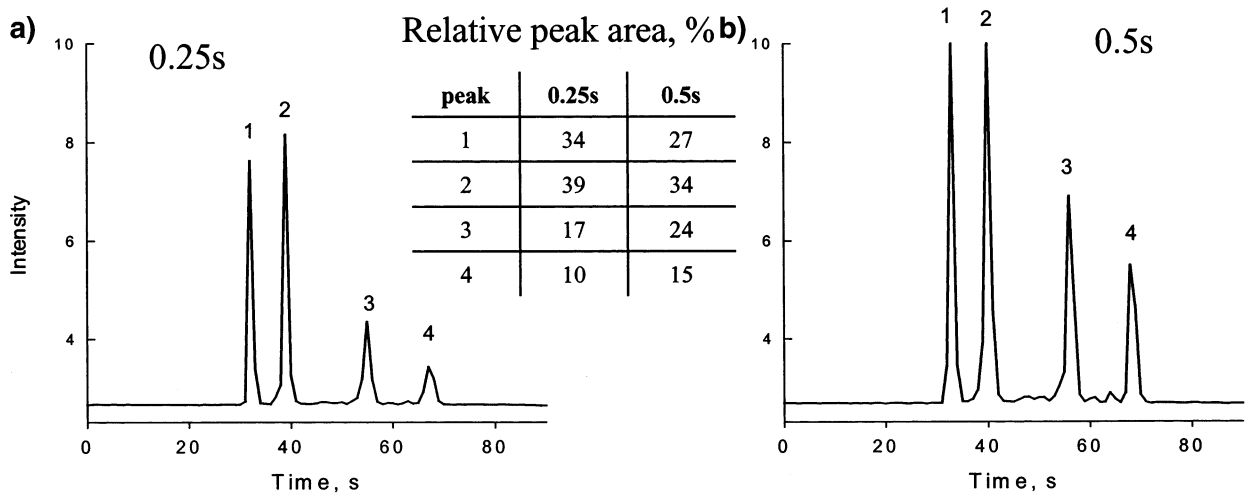

peak	0.25s	0.5s
1	34	27
2	39	34
3	17	24
4	10	15

Figure 3. Separation of FITC-labeled synthetic peptide mixture following 0.25-s (a) and 0.50-s (b) electrokinetically based gated injection onto a C_{18} AMPS-modified microcolumn. Analyte concentrations: 1 + 2 = (1/3)4 = 2.5×10^{-5} M. Other conditions are as in Figure 2.

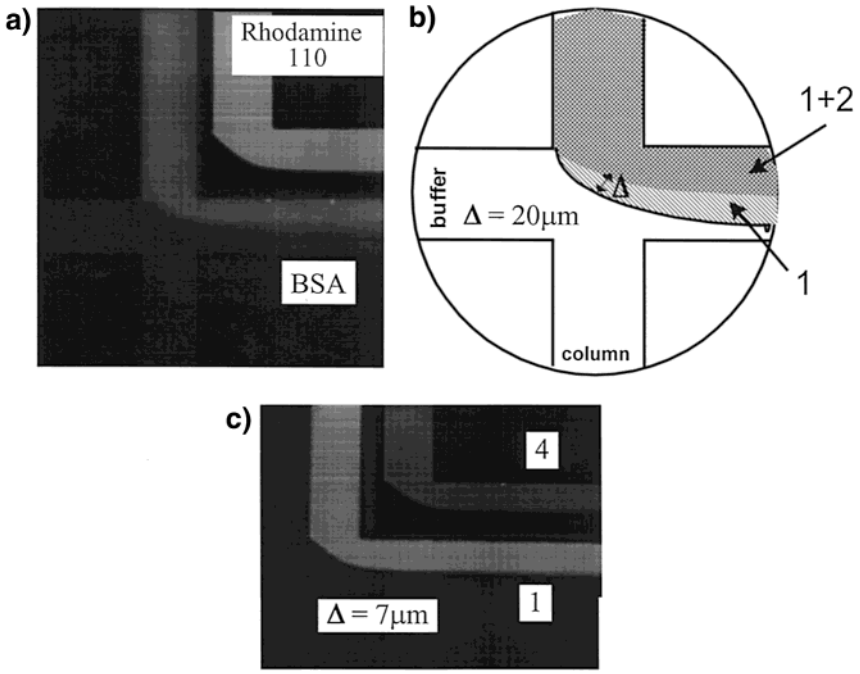

Figure 4. (a) Individual flow profiles taken by CCD camera of FITC−BSA and rhodamine 110, (b) combined flow profile of the two compounds with different electrophoretic mobilities, and (c) individual flow profiles of compounds 1 and 4 taken by CCD camera, as in Figure 2.

be responsible for these differences. This phenomenon will be referred to henceforth as transradial electrokinetic selection (TREKS).

This is the first time TREKS has been described in the context of sampling bias. The turning radius of an analyte in a channel junction of width (w) can be estimated through its Peclet number (Pe) where

$$Pe = \frac{v_i w}{D_i} \tag{3}$$

Assuming the diffusion coefficient (D) of these small peptides to be 10^{-6} cm^2/s, the Peclet numbers for peptides 1 and 4 were estimated to be ~800 and 450, respectively. Moreover, with small molecules, the term w/D_i will be a constant, and the Peclet number ratio for two analytes will be

$$\frac{Pe_1}{Pe_2} = \frac{v_1}{v_2} \tag{4}$$

The analyte with the highest Peclet number will have a larger turning radius and be closer to the column as a result of its higher velocity. This will cause a second level of sampling bias in the channel junction as voltage is switched during gated flow injection. If analytes of the same concentration differ 2-fold in electrokinetic transport velocity, the resulting sample aliquot would contain these analytes in a concentration ratio greater than 1:2 because of the impact of TREKS. This difference would be even more significant in biological samples for which ratios in velocity among analytes can be 10 or more.

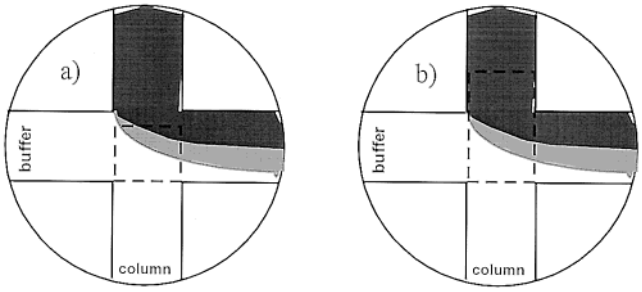

Figure 5. Illustration of an electrokinetically gated injection of 0.25 s (a) and 0.50 s (b) duration showing the approximate length of sample injected (dashed boxes). The resulting chromatograms are seen in Figure 3.

The dashed boxes in Figure 5a,b show the approximate area injected during the 0.25- and 0.50-s switching times used to execute the gated injection that produced the chromatograms seen in Figure 3a,b. The dashed box height was equal to the distance that the least retained fastest migrating compound migrates during the allotted injection time at the applied voltage. It is seen in Figure 5a that the sample would experience two levels of electrokinetic selection in the channel junction, one from the normal bias described in eq 1 and another from TREKS. As the switching time increases and the zone sampled becomes larger, the contribution of TREKS will be smaller (Figure 5b). When injection time is increased beyond 0.50 s, the impact of the small TREKS zone will become minor, and the total sampling bias for the system will approach that described in eq 1. The unfortunate disadvantage of having to increase the sample volume to overcome the TREKS effect is that separation efficiency can be diminished. Furthermore, there are still two possible levels of selection. A less discriminating sample inlet system is needed.

Diffusion Based Injection. Gated flow injection is based on electrokinetically driven transport of analytes from the sample inlet channel into the separation channel. It has been shown above that an alternative to this process is to drop the potential to 0 on all electrodes in the system and allow analytes to diffuse into the separation channel before reapplying potential to electrodes I and II in Figure 1. Diffusion between the juxtapositioned streams seen in Figure 6a at 0 potential is the basis for this type of sample introduction. Entry of analytes into channels that transport buffer from reservoir I to IV is seen to occur within seconds. After 0.75 s, the sample has already diffused through the channel cross and into the separation channel (Figure 6b), and in 3 s, a plug with a flat leading edge toward the separation column has formed (Figure 6c). When the desired amount of sample has diffused into the separation channel, potentials are reapplied to electrodes I and II, pushing the sample plug into the separation column (Figure 6d).

It is clear that analyses of the same mixture with diffusion-based and electrokinetic injection produce completely different peak area ratios (Figure 2a,b). Later eluting peaks are seen to be more abundant in diffusive injection. Although electrokinetic selection from the sample reservoir and the TREKS phenomenon are both at work in diffusive injection, the second level of electrokinetic selection that occurs in gated flow injection will be absent in the diffusive injection mode. Elimination of electrokinetic selection at any level will diminish the loss of analytes showing negative electrophoretic mobility, as observed.

But diffusive injection will probably have its own unique kind of sampling bias. According to Fick's laws,

$$J_x = -D\frac{dc}{dx} \tag{5}$$

where J_x is the flux of an analyte in direction x away from an analyte source measured in mole/(cm^2/s), D is analyte diffusion coefficient in cm^2/s, C is analyte concentration in mole/cm^3, and dc/dx is the concentration gradient. This means that differences in the diffusion coefficients of analytes will impact the relative amount of analytes diffusing into the channel junction, and diffusive injection will thus have a diffusion bias in sampling. When sample components are of similar size, as in the case of tryptic peptides, analyte diffusion coefficients will be similar, and the diffusion bias will be small. But the diffusion bias will be large in the case of analytes that vary a 100-fold in diffusion coefficient, such as proteins and peptides.

The amount of sample injected in diffusive sampling is regulated through the diffusion time, that is, the time the systems sets at 0 potential. Figure 7a is a series of snapshots taken by a CCD camera, showing the increase in plug length with increasing injection time. The length of the plug grows from 23.8 μm after 2-s diffusion time to 84.6 μm with a 10-s diffusion time. The volumes of injected plugs were computed to increase from 12.8 to 45.5 pL during this time. Peak area of the injected peptides

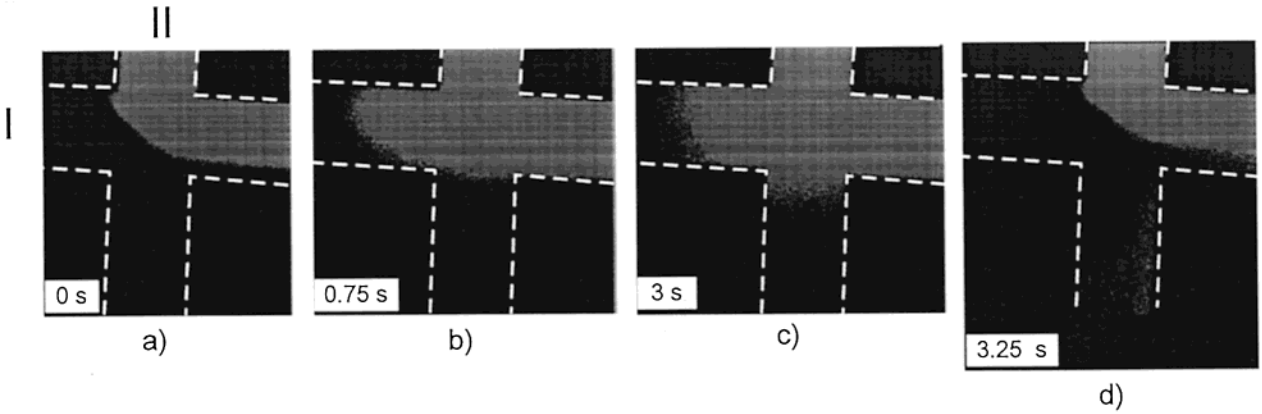

Figure 6. Frames taken by a CCD camera at 0 (a), 0.75 (b), and 3 s (c) during a 3-s diffusion-based injection and the resulting sample plug injected (d). Sample and mobile phase conditions were the same as in Figure 2.

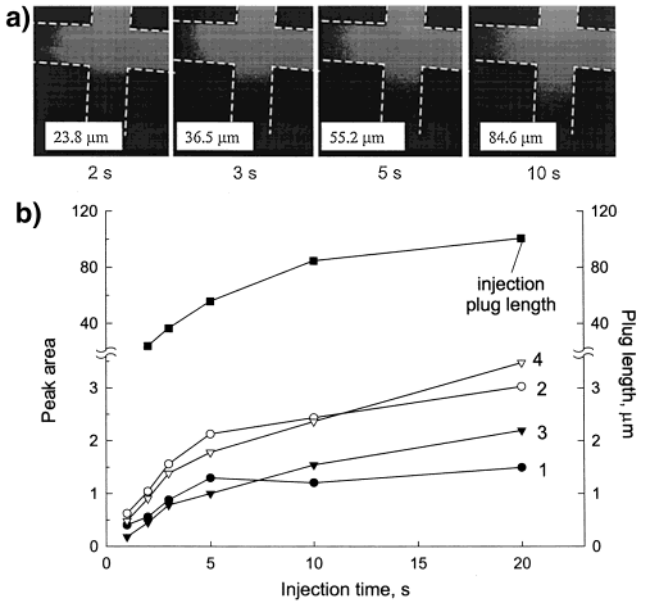

Figure 7. (a) Fluorescent microscopy images of sample plugs after 2-, 3-, 5-, and 10-s diffusions into the separation channel. The corresponding injection plug length is shown. (b) Plot of peak area and injection plug length versus injection time for diffusion-based injection. Sample and mobile phase conditions were the same as in Figure 2.

Table 1. Comparison of Retention Time and Peak Area Reproducibility[a] for FITC-Labeled Peptides in Electrokinetic and Diffusion Based Injection[b]

injection type	retention time	peak area
0.25-s electrokinetic	2.13	15.3−17.9
5-s diffusion	1.51	1.10−2.33

[a] RSD, %. [b] $n = 20$.

increased in proportion to the square root of time, as would be predicted by theory (Figure 7b).Twenty subsequent separations after 5-s-long diffusion-based and 0.25-s-long electrokinetic injections were performed to evaluate the reproducibility of both injection procedures. The relative standard deviation (RSD) of retention time and peak area for diffusion injection (Table 1) is comparable to values previously reported for gated injections on a quartz chip.[11] Similar data for gated injection with modified PDMS chips were not reported. The poor RSD for peak area and retention times in electrokinetic injection was, perhaps, due to the difficulty of controlling injection flow in PDMS or the heterogeneity of its surface.[14] The injection procedure proposed in this paper is simpler than using the active potential control method.[13]

CONCLUSIONS

It is concluded that multiple levels of electrokinetic sampling bias are at work in the electrokinetically gated flow injection and that this injection scheme is unreliable for the introduction of complex samples with analytes of widely differing electrophoretic mobility. One form of bias is the normal electrokinetic selection seen in all forms of electrokinetic sampling. A new form of sampling bias in gated flow injection was also observed that originates from transradial electrokinetic selection (TREKS). This phenomenon results from differences in turning radii of analytes as they pass through a channel junction and make a right angle turn toward the waste reservoir. The path and turning radius of an analyte through the channel junction depends strongly on their electrophoretic mobility. The overall sampling bias in gated injection is the sum of these two forms of bias. It is time-dependent and results in a large sample concentration misrepresentation.

It is further concluded that diffusion-based sample injection is superior to electrokinetic gated flow injection. This conclusion is based on the fact that diffusion of analytes from a sample stream into the separation channel at 0 potential produces less sampling bias than by electrokinetic injection. Moreover, this new scheme still allows variable sample volume (12−45 pL) with a high degree of reproducibility in retention times (RSD < 1.5%) and peak areas (RSD < 2.3%). Although diffusion-based injection has a diffusion coefficient bias in sampling, this is only a problem when analytes vary in size. Diffusion-based injection will be most useful when analyzing complex samples with analytes that differ widely in electrophoretic mobility, such as peptides.

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