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Gradient Elution in Microchannel Electrochromatography

Michael W. L. Watson,[†] Jared M. Mudrik,[†] and Aaron R. Wheeler*[,†,‡,§]

Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario, M5S 3H6, Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, University of Toronto, Toronto, Ontario M5S 3G9, and Banting and Best Department of Medical Research, University of Toronto, 112 College Street, Toronto, Ontario, M5G 1L6

There is great interest in using microfluidic channels packed with a stationary phase for chemical separations of complex mixtures. A key advantage of such techniques is the use of electroosmotic flow (EOF), controlled simply by applying electrical potentials between reservoirs. A disadvantage for this technique, however, is a lack of compatibility with gradient elution separations. This limitation arises from the dependence of EOF velocity on run buffer content (including the concentration of organic modifier). Here, we introduce a method for implementing gradient elution in electrochromatography in which multiple run buffers are velocity-matched, such that the elution profile resembles that found in conventional HPLC. This method is driven entirely with EOF, meaning that pumps, valves, and pressure fittings are not required. The method was validated by application to separations of peptide standards and protein digests. These results suggest that microfluidic electrochromatography may be compatible with a wide range of applications that have previously been unexplored.

Microchannels facilitate fast analyses with higher resolution, higher efficiency, and lower reagent consumption relative to their macroscale counterparts. An especially attractive feature of microfluidic separation systems is the capacity to control samples and run buffers by electroosmotic flow (EOF), with no need for external mechanical pumps, valves, or pressure fittings. The simplest (and most common) microfluidic separations rely on liquid-phase electrophoresis in open channels for separation of charged analytes. While electrophoresis is useful for some applications, chromatography, which can facilitate separation of complex mixtures on the basis of a wide range of phenomena, is better suited for many others. In recognition of this need, there is a growing trend toward developing ever more sophisticated chromatography techniques in microfluidics.

Chromatography methods in microchannels can be broadly classified by the nature of flow: pressure-driven or EOF. In the former, external pumps are connected to devices to drive fluid through columns in microchannels, as in the Agilent HPLC-Chip.[1] These methods mirror HPLC (with pumps, fittings, and valves) and hence compromise some of the advantages of microfluidics. The latter class of techniques, called electrochromatography, relies on EOF to drive buffers and analytes through a stationary phase. This technique capitalizes on many of the advantages of microfluidics (e.g., no external pumps or valves); however, it is not directly translatable to many useful and important techniques used in macroscale chromatography, such as gradient elution.

Here, we report a new method for microfluidic electrochromatography, in which an acetonitrile gradient is generated on-chip for elution of analytes through a reversed phase column. The guiding principle of this work was to develop a microfluidic system that is comparable to the common gradient elution methods used in HPLC for proteomics.[2] The work reported here joins a small group of studies that have explored gradient elutions in microchannel electrochromatography and related techniques. For example, Kahle et al.[3] and Sakai et al.[4] demonstrated gradient elution through a stationary phase immobilized in a capillary. These systems are functional but far from the microfluidic ideal, as they require external pumps, valves, and pressure fittings. The Ramsey group at the University of North Carolina demonstrated gradient elution in microchannels in open tubular chromatography (OTC) mode.[5,6] While an important first step, the limited surface area of stationary phases in OTC makes it impractical for separating complex mixtures. Finally, the same group recently published a conference proceedings paper describing gradient elution in a microchannel bearing a traditional stationary phase.[7] This work is an important milestone; however, it highlights a significant problem in marrying electrochromatography to gradi-

* Corresponding author. E-mail: awheeler@chem.utoronto.ca. Phone: 416-946-3864. Fax: 416-946-3865.

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[‡] Institute of Biomaterials and Biomedical Engineering.

[§] Banting and Best Department of Medical Research.

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ent elution: EOF velocity is highly dependent on run buffer composition (including acetonitrile concentration), which makes programming mobile phase elution profiles that mimic those used in HPLC a significant challenge.

Here, we introduce a technique for solving for the EOF velocity problem: the salt concentration is titrated in a pair of run buffers containing different concentrations of acetonitrile such that linear combinations of the two buffers maintain constant velocity. The resulting method, which does not require pumps, valves, or fittings, generates chromatograms that are largely consistent with what is expected in HPLC. The stationary phases used here were formed from porous polymer monoliths (PPMs) fabricated *in situ* by photopolymerization of acrylate monomers.[8−12] However, we anticipate that this technique will be compatible with many types of stationary phase, including silica monoliths,[13−16] packed beds of beads,[17−19] microfabricated pillars,[20,21] or PPMs formed from various other functional groups.[22−24] As the first technique of its kind, we believe this to be an important step forward for making electrochromatography a better match for a wider range of applications.

EXPERIMENTAL SECTION

Reagents and Materials. Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (Oakville, ON) and used without further modification. All buffers were formed using deionized water (diH_2O, 18 MΩ cm) and were filtered with nylon syringe filters from Millipore (Billerica, MA, 0.2 μm pore diameter) and sonicated (5 min) prior to use. Trimethoxy silyl propyl acrylate (TMSPA) was purchased from TCI America (Portland, OR). Nanoports, Luer-to-Microtight fittings and PEEK transfer capillaries were purchased from Upchurch (Oak Harbor, WA). PDMS for reservoir manifolds was from Dow Corning (Midland, MI). Acetonitrile (ACN), methanol (MeOH), and ethanol (EtOH) were from ACP (Montreal, QC). Fluorescein isothiocyanate-labeled casein (FITC-Casein) was purchased from Invitrogen (Burlington, ON).

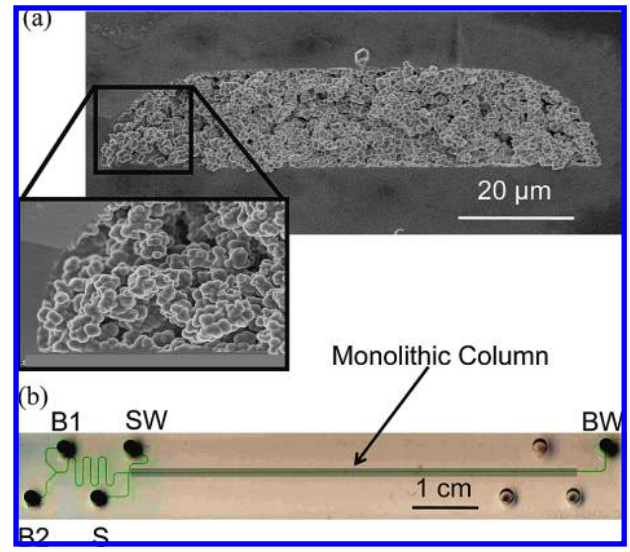

Figure 1. Pictures of a polymer monolith and a device. (a) An SEM image of a monolith shows the structure of the polymer, which is covalently anchored to the wall (inset). (b) The device (filled with green dye for visualization) has three inlets (B1, B2, S) and two outlets (SW, BW). The shaded region shows the position of the stationary phase.

Fluorescently labeled peptide standards and protein digests were used as model analytes. Peptide standards were labeled and purified using a new method we call the "albumin-mop." Briefly, standards of bradykinin, oxytocin, angiotensin I, II, III, and IV, and leucine enkephalin (50 μM) were labeled with FITC (1 mM) in borate buffer (50 mM, pH 9) overnight. Unreacted FITC was then removed by mixing with bovine serum albumin (BSA, 69 kDa, 10 mg/mL final concentration) overnight and then passing the mixture through two Millipore 10 000 Da molecular weight cutoff centrifugal filters (60 min, 14 000*g*), collecting the filtrate each time. Labeled standards were stored in the dark in a freezer (−20 °C) until use, at which time they were thawed and diluted in run buffer to the desired concentration. Protein digests were prepared by incubating FITC-Casein (50 μg/mL) with trypsin (500 μg/mL) in borate buffer (50 mM, pH 9, room temperature, >6 h). Following digestion, the sample was diluted into run buffer (1:1) for separation.

Device Preparation. Two types of wet-etched glass microfluidic devices were used, both with 20 μm deep, 100 μm wide channels: (1) a custom five-reservoir design (shown in Figure 1) from Micralyne (Edmonton, AB), courtesy of CMC Microsystems (Kingston, ON) and (2) an off-the-shelf four-reservoir cross design (~0.6 cm inlet reservoirs-to-cross, ~3.4 cm cross-to-buffer waste) from Caliper Life Sciences (Hopkinton, MA). As shown in Figure 1b, the custom design (used in most experiments) has two buffer inlets, one sample inlet, and two outlets (sample waste and buffer waste). A serpentine channel (~4.75 cm) separates the buffer inlets from the injector/separation column; this length is sufficient for complete mixing of the buffer constituents (i.e., water, acetonitrile, and sodium borate) at the velocities used here. The separation channel runs from the 200 μm double-tee injector to the buffer waste outlet and is ~7.5 cm long.

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Prior to forming monoliths, the devices were pretreated with TMSPA to anchor the stationary phase to the channel walls. Reagents used in this process were propelled by a syringe pump (Harvard Apparatus, Holliston, MA) through a Luer-to-Microtight fitting mated to a PEEK transfer capillary, mated again to a Nanoport assembly, which was held in place on the device using a homemade pressure jig. The channel walls were first activated by applying base (200 mM NaOH, 2 μL/min, 1.5 h) and then rinsed with diH_2O and MeOH. Methanolic TMSPA solution (20 vol %) was applied (2 μL/min, 1.5 h) followed by a MeOH rinse and drying under nitrogen. After pretreatment, the pressure fittings were removed, and a PDMS manifold was plasma-bonded to the chip to form solvent-resistant reservoirs.[25] Finally, channels were dried overnight in an oven (70 °C).

Monolith Preparation. Porous polymer monoliths (PPMs) were fabricated by *in situ* photopolymerization of a casting solution in dry, pretreated channels. Casting solutions comprised a mixture of acrylate monomers, photoinitiator, and porogenic solvent, similar to what was reported by Throckmorton et al.[11] A monomer/photoinitiator mixture was formed by measuring the solid reagents, 2,2-dimethoxy-2-phenylacetophenone (DMPA, 2.5 mg) and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS, 1.2 mg) into an amber glass vial, followed by liquid reagents, butyl acrylate (BA, 278.5 μL), 1,3-butanedioldiacrylate (BDDA, 150 μL), lauryl acrylate (LA, 69 μL), and TMSPA (2.5 μL). A porogenic solvent comprising aqueous phosphate buffer (5 mM, pH 6.8), ACN, and EtOH (1:3:1 ratio, respectively) was formed and then combined with the monomer/initiator mixture (1:2 monomer/initiator mixture−porogenic solvent) to form the casting solution.

The inclusion of AMPS in the casting solution facilitates EOF in the monolith; however, in most cases (as above) the concentration was kept low at 0.25 wt %. In this case the monolith is predominately hydrophobic, such that it behaves as a reversed-phase column. In some cases, however, charge density was varied by using different amounts of AMPS (0.10, 0.50, and 1.00 wt %); in such cases, the amount of BA was varied in concert (AMPS/BA: 0.6 mg/279.3 μL; 2.5 mg/277 μL; 5.0 mg/275 μL) to maintain comparable monomer density. We note that reproducible monolith formation is dependent on using dry AMPS; thus, it is imperative to store it in a desiccator.

Monolith formation was initiated by pipetting casting solution (20 μL) into the buffer outlet reservoir such that it filled the channel network by capillary action. The flow was balanced after filling by dispensing equivalent volumes of casting solution into the remaining reservoirs and covering them to prevent evaporation. Electrical tape on the bottom surface of each device served as a crude photomask to form monoliths beginning ~50 μm downstream of the injectors (monoliths were ~7 cm long in custom devices and ~2.5 cm long in Caliper devices). Devices were exposed to UV radiation (100 W, 365 nm, 5 min) from below using a lamp (UVP, Upland, CA); during polymerization, a small fan kept the lamp and device cool. After polymerization, the reservoir solutions were replaced with phosphate buffer (4 mM, pH 6.8, 30% ACN) and unreacted casting solution was driven out of the channels by EOF (~150 V/cm, 120 min).

After fabrication, some PPMs were characterized using a scanning electron microscope (SEM, Hitachi High Technologies America, Pleasanton, CA). In these experiments, devices were prepared by drying in an oven (70 °C, overnight) and then freezing (−20 °C, 2−3 h). Upon removal from the freezer, the devices were scored with a diamond scriber to break into segments (~0.5 cm × 0.5 cm) which were then mounted in custom holders and coated with graphite using a high-vacuum carbon coater (Emitech, Ashford, Kent, U.K.).

After being used in electrochomatography experiments, monoliths were removed by thermal incineration using methods similar to those reported by Throckmorton et al.[11] Such monoliths were flushed with run buffer by EOF (~150 V/cm, 30 min), after which the reservoirs were removed and the device was placed in a muffle furnace (Barnstead, Dubuque, IA). The furnace was ramped from room temperature to 550 °C (10 °C/min) and then held at that temperature for at least 2 h before allowing it to cool. The channels were filled with concentrated sulfuric acid (by capillary action) after reaching room temperature and allowed to incubate overnight and then rinsed with water and dried before reuse.

Buffer Velocity. EOF velocities were estimated using the current monitoring method, evaluating similar run buffers with different conductivities.[26] Briefly, in each experiment, the separation channel was filled and equilibrated with a high-conductivity buffer (e.g., 20 mM borate) by applying a potential between the buffer inlet(s) and the buffer waste outlet (~500 V/cm, 5 min). After equilibration, the contents of the inlet reservoir(s) were replaced with a low-conductivity buffer (e.g., 10 mM borate) and the same potentials were applied to fill the column with the new buffer. During these measurements, the potential drop across a resistor (100 kΩ) connected in series between the buffer waste reservoir and ground was recorded using an A-D card and a PC running LabVIEW (National Instruments, Austin, TX). The EOF velocity was estimated as the column length divided by the time required for the current to decrease to an inflection, representing the time at which the original buffer was completely replaced.

Current monitoring was used to measure EOF velocities for two applications. In the first application, using off-the-shelf Caliper devices, the effects on flow rate of (a) charge on the monolith and (b) organic modifier concentration in the run buffer were evaluated. In these experiments, monoliths were formed from 0.10, 0.50, and 1.00 wt % AMPS (as described above), and the high and low conductivity run buffers for current monitoring were 20 and 10 mM borate (pH 9), respectively (each containing 10, 30, or 60% ACN). Replicate analyses ($n = 5$) for each condition were evaluated to determine the effects on EOF velocity in isocratic elution experiments.

In the second application, current monitoring was used to determine a matched-velocity two-buffer system for gradient elution in custom devices (Figure 1) bearing monoliths formed from 0.25 wt % AMPS. In iterative experiments, the borate concentration in a 2% ACN buffer was systematically increased and the velocity was estimated using a pair of buffers with a 10% difference in borate concentration (e.g., the velocity of a 2% ACN, 100 mM borate buffer was estimated using a 2% ACN, 100/90 mM borate buffer pair). By this method, a pair of buffers containing low ACN (180 mM borate, pH 9, 2% ACN) and high

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Run-to-Run Variability

	Ret. Time (%RSD)	Rel. Area[a] (%RSD)	Efficiency N (x 10^4)
Ang III	1.24	2.16	4.44
Ang II	0.63	2.40	2.86
Ang IV	0.98	/	/

Device-to-Device Variability

	Ret. Time (%RSD)	Rel. Area[a] (%RSD)	Efficiency N (x 10^4)
Ang III	1.03	2.15	4.75
Ang II	1.71	2.59	3.16
Ang IV	1.60	/	/

[a]*Relative to angiotensin IV.*

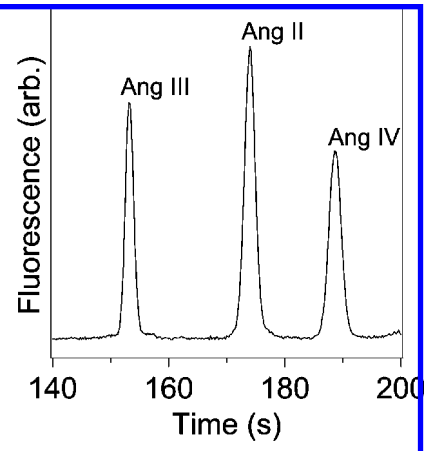

Figure 2. Column reproducibility was evaluated with replicate injections of FITC-labeled angiotensins to analyze variability of retention time and peak area, as well as to determine average efficiencies. Run-to-run variability was assessed on a single column with replicate ($n = 5$) injections. Device-to-device variability was assessed with three individually prepared columns with replicate injections ($n = 15$). The right panel is a typical electrochromatogram for the separation of the three angiotensins. All separations were performed using the same run buffer (100 mM borate, pH 9, 10% ACN).

ACN (20 mM borate, pH 9, 60% ACN) were determined, each of which had an EOF velocity of ~0.8 mm/s when driven at 245 V/cm.

Electrochromatography. Separations experiments were performed in the custom devices (Figure 1) bearing monoliths (most formed with 0.25 wt % AMPS). Electrokinetic flow was programmed and driven using a high-voltage sequencer (LabSmith, Livermore, CA) and associated software. Analytes were loaded onto the double-tee and then the column by pinched injection,[27] and isocratic separations were driven by applying 3.0 kV between the buffer inlets (containing the same run buffer) and buffer waste, resulting in an electrical field of 245 V/cm. For gradient separations, the two buffer inlets (containing different run buffers) were biased using matched and linearly increasing/decreasing voltage programs ranging between 3.0 and 2.8 kV. Analytes were detected 7 cm downstream from the injection tee by laser-induced fluorescence using an inverted microscope (Olympus IX-71) mated to an argon ion laser (Melles Griot, Carlsbad, CA). The 488 nm laser line (20 mW) was focused into the channel using an objective (60×); the fluorescent signal was collected by the same lens and filtered optically (536/40 nm band-pass and 488 nm notch filter) and spatially (500 μm pinhole), and imaged onto a photomultiplier tube (Hamamatsu, Bridgewater, NJ). PMT current was converted to a voltage using a picoammeter (Keithley Instruments, Cleveland, OH) and then collected using an A-D converter and a PC running a custom LabVIEW (National Instruments) program.

Monolith variability was assessed by analyzing isocratic electrochromatograms of FITC-labeled angiotensin II, III, and IV on PPMs formed with 0.5 wt % AMPS. Data were analyzed using PeakFit software (SeaSolve Software Inc., Framingham, MA) to determine peak area, retention time (t_R), peak width at half-max ($W_{1/2}$), and the number of theoretical plates (N).

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$$

Data generated from a single column ($n = 5$ injections) were compared to assess the run-to-run variability, and data ($n = 15$ injections) generated from three columns (formed in three different devices) were pooled and evaluated to assess column-to-column reproducibility.

RESULTS AND DISCUSSION

Column Preparation and Characterization. *In situ* fabrication makes polymer monoliths an attractive option for stationary phases in microfluidic devices. The acrylate-based polymers used in this work were particularly convenient in that (a) photopolymerization facilitated easy patterning (i.e., some channels are filled while others remain open), and (b) by incineraton of the columns after use, the device lifetimes were significantly extended (e.g., some of the devices used here were recycled with new monoliths more than 10 times). In addition, the use of an aqueous porogen facilitated manipulation by EOF, which is beneficial because world-to-chip pressure interfaces for microchannels are awkward to form. Pictures of a monolith and a device are shown in Figure 1. The SEM demonstrates that monoliths possess a high surface area to volume ratio and are covalently anchored to the channel walls. Figure 1b is a picture of a representative device used in the study. The chip contains two buffer inlets (B1 and B2), a sample inlet (S), a buffer outlet (BW), and sample outlet (SW).

As depicted, the stationary phases used here were positioned in the separation channel between the injection tee and buffer outlet. This stands in contrast to previous work[11] in which the entire channel network was filled with stationary phase. In our devices, because the surface charge densities are different in the open and polymer-filled regions of the channels, there are likely pressure-driven flows (in addition to EOF). This is tolerable, as our goal was to develop HPLC-like separation functionality (i.e., gradient elution), not to generate perfect EOF profiles.

A common criticism of the use of polymer monolith stationary phases is poor reproducibility (relative to packed beds of beads); indeed, some have likened PPMs to "black magic."[28] To charac-

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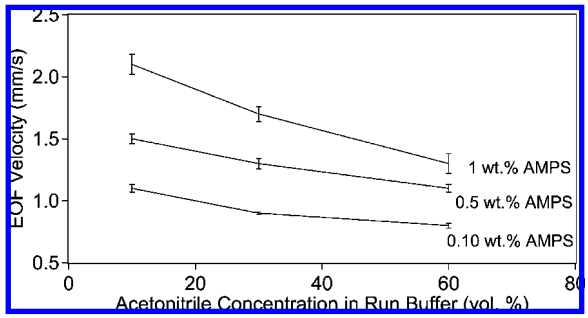

Figure 3. Graph depicting the effects of surface charge and ACN concentration in the run buffer on EOF velocity. As shown, increased surface charge density results in higher EOF velocity, and increased ACN concentration results in lower EOF velocity. Error bars are ±1 SD.

terize the reproducibility of the monoliths used here, we evaluated run-to-run and column-to-column variability using replicate injections of FITC-labeled peptides. Figure 2 reports the results of this analysis, including variability in retention time (absolute) and peak area (relative) as percent relative standard deviation (% RSD), as well as the average number of theoretical plates. As shown, the run-to-run variability (measured in five replicate injections) for retention time (RSD < 1.25%) and peak area (RSD < 2.5%) is very low, and the devices exhibit efficiencies expected for such systems (N = 20 000−40 000 plates). Likewise, the column-to-column variability (measured in five replicate injections each in three different columns on three different devices) in retention time (RSD < 1.7%) and peak area (RSD < 2.6%) was low and had similar efficiencies. These results suggest that with careful attention to fabrication, PPMs can be highly reproducible, making them appropriate for use in demanding separation experiments.

EOF Velocity. We used the current monitoring method[26] to characterize the electroosmotic flow velocities in PPM stationary phases. Because the channels were partly open and partly filled with monolith, the measured velocity represents an average value for the entire flow path. As far as we are aware, this is the first report of applying this type of analysis to microchannels containing a stationary phase.

In these tests we evaluated two properties: surface charge density and organic modifier concentration. These properties are important, as EOF velocity is proportional to double layer thickness (which varies by surface charge and buffer composition). Devices bearing monoliths with different surface charge densities were evaluated using three buffers, each with different ACN concentration. Each condition was analyzed in replicates (n = 5), and the data is shown in Figure 3. As expected, monoliths with higher AMPS content (i.e., more negative charge density) supported greater cathodic EOF velocity. Likewise, buffers with higher organic modifier concentration resulted in reduced EOF velocity. These data are useful, as they established a baseline for developing two-buffer systems with identical velocities for gradient elution.

Gradient Elutions. Gradient elution in reversed-phase HPLC is typically implemented by varying the pressures applied to two solvents containing different concentrations of organic modifier. In such systems, the pressures driving these solvents are ramped up and down relative to each other such that the overall flow rate remains approximately constant. An analogous gradient elution method in electrochromatography might be implemented by varying the potentials driving electrokinetically pumped run buffers containing different concentrations of modifier (but are otherwise identical). Unfortunately, because EOF velocity is strongly dependent on run buffer modifier content (as shown in Figure 3), the overall flow rate in such a system is likely to vary dramatically during the course of an experiment, complicating the analysis. To overcome this problem, we developed a new method in which two buffers containing different amounts of organic modifier are velocity-matched, such that the overall flow rate remains constant during gradient elution.

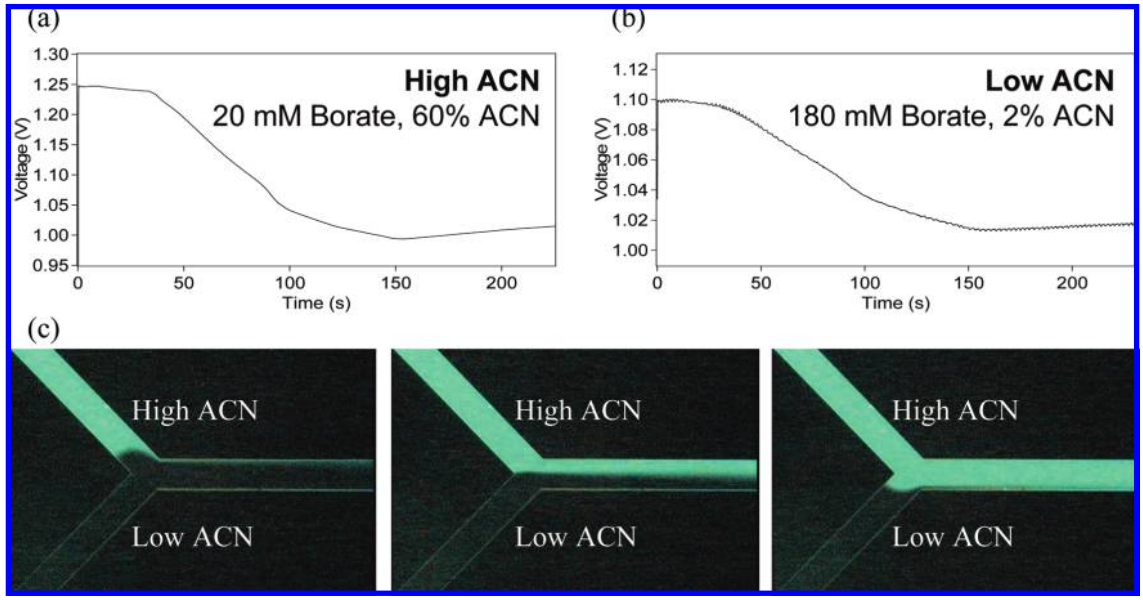

Figure 4. Current monitoring traces and pictures depicting the velocity-matched gradient elution strategy. (a,b) Current monitoring traces for high-ACN and low-ACN buffers; as shown, they have matched inflection points, indicating matched EOF velocities. (c) Sequence of frames from a movie (left-to-right) depicting run buffer generation for 2% ACN (frame 1), 31% ACN (frame 2), and 60% ACN (frame 3). The high-ACN buffer included 1 μM fluorescein for visualization.

In this work, we targeted 2%−60% ACN as the desired mobile phase gradient (appropriate for the reversed-phase chromatographic media used here) and developed the elution procedure in three steps. First, a slow-moving, 60% ACN run buffer velocity was measured to be approximately 0.8 mm/s (Figure 4a). Second, the velocity of a complementary 2% ACN run buffer was empirically tuned to the same velocity by increasing the salt concentration (Figure 4b). Third, the high-voltage sequencer software was programmed to linearly ramp the low-ACN buffer from 3.0 to 2.8 kV (relative to BW), while the high-ACN buffer was ramped from 2.8 to 3.0 kV. Figure 4c shows a series of images collected at the junction of the two buffer channels while implementing this voltage sequence (the high-ACN buffer contains 1 μM fluorescein for visualization). The overall flow rate remains approximately constant during this process, and the time required for the buffer to travel through the serpentine channel (~59 s) is sufficient for complete mixing by diffusion.

In developing this technique, we considered several strategies for implementing gradient elution: (a) noncontrolled (varying) flow rate; (b) constant flow rate facilitated by applying a lower electrical field to the high-velocity buffer; and (c) constant flow rate facilitated by using velocity-matched buffers (the method ultimately adopted). The first approach (a) is undesirable, as it negates the advantage of using gradient elution: as ACN concentration increases, the mobile phase velocity decreases, lengthening the run-time (as reported previously[7]). The second approach (b) is also undesirable, as the separation efficiencies in EOF-driven systems are highest when using the maximum (non-Joule-heating) electrical field. The third approach (c) is a good compromise: the overall buffer velocity is constant, and the highest possible electrical field (using our instrument) is applied. We acknowledge that this method is not universal; significant changes in buffer content would require recalibration of the individual velocities. However, despite this limitation, as this is the first such method ever reported, it is a useful starting point for future work.

The velocity-matched buffers described above were used in separations of derivatized peptide standards with varying driving voltages to create ACN gradients. Representative electrochromatograms are shown in Figure 5. The data in parts a and b of Figure 5 demonstrate that if the gradient slope is too high, the analytes are poorly resolved. When a lower slope in the gradient

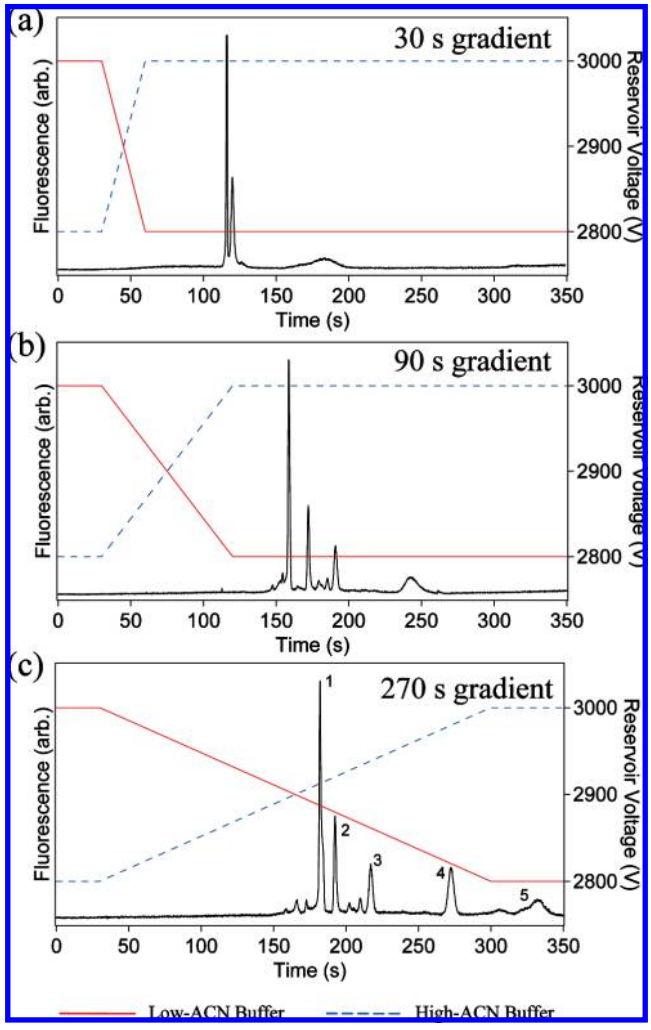

Figure 5. Electrochromatograms of FITC-labeled peptide standards (40 nM each; 1 = bradykinin, 2 = oxytocin, 3 = angiotensin I, 4 = angiotensin IV, and 5 = leucine enkephalin). Gradients from low ACN (2%) to high ACN (60%) were generated over varying times (30, 90, or 270 s). Reservoir voltages on the low-ACN (red, solid) and high-ACN (blue, dashed) buffer inlets were varied as indicated.

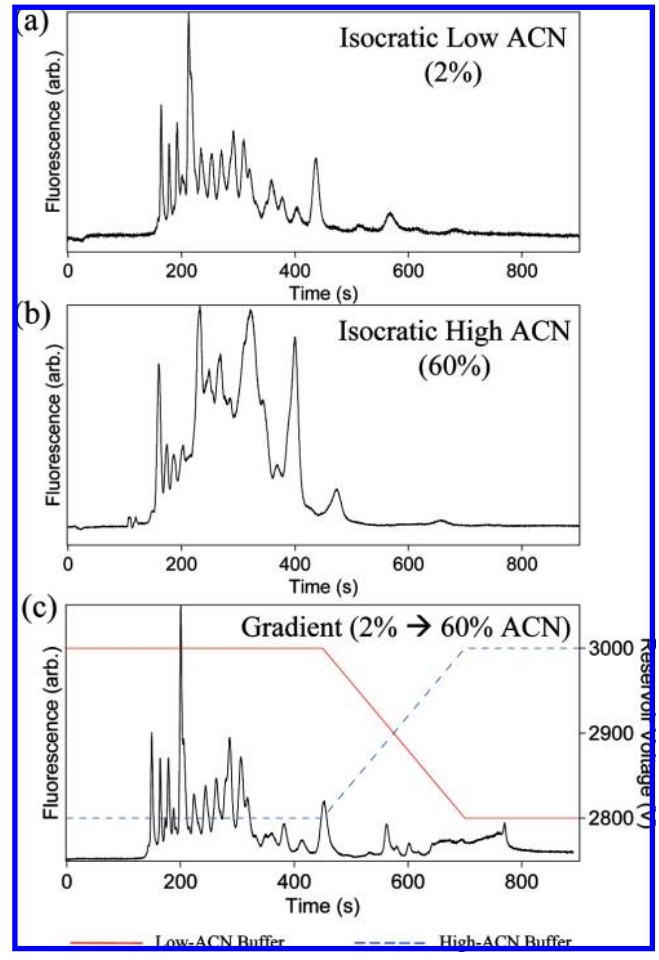

Figure 6. Electrochromatograms of a tryptic digest of FITC-labeled casein (25 μg/mL). Isocratic mode separations with low-ACN buffer (a) and high-ACN buffer (b) were not optimal, resulting in nonelution of some analytes (a) or poor resolution (b). A gradient elution (c) of 2%−60% ACN combined the best of both buffers, allowing for good resolution, with elution of all of the analytes. The gradient was formed by varying the low-ACN (red, solid) and high-ACN (blue, dashed) buffer inlet voltages as indicated.

is used, as in Figure 5c, all of the peaks are resolved. Note that as the slope of the gradient is reduced, the first peak elutes at progressively longer retention times. This mirrors the behavior of reversed phase HPLC; moderately hydrophobic analytes remain bound to the column until the mobile phase contains a higher concentration of ACN. The opposite trend is observed when run buffers with different EOF velocities are used.[7]

Gradient elution is a workhorse for applications in which complex mixtures must be separated (e.g., shotgun proteomics[2]). To evaluate the compatibility of the method described here with such samples, electrochromatography was used to analyze a tryptic digest of FITC-Casein (Figure 6). Isocratic electrochromatograms were also generated to illustrate the advantages of gradient elution. For example, a 2% ACN run buffer in isocratic mode (Figure 6a) yields good peak resolution for fast-eluting analytes, but the more hydrophobic analytes elute very slowly (and some do not elute at all). In contrast, a 60% ACN buffer in isocratic mode (Figure 6b) drives all analytes off of the column quickly; however, the peaks are poorly resolved. A gradient elution from 2−60% ACN combines the best of both buffers, yielding good peak resolution and eluting all analytes off of the column in reasonable time.

CONCLUSION

In this work, we introduced the use of the current monitoring method to measure EOF velocity in electrochromatography. This technique was used to determine a pair of velocity-matched run-buffers for implementing constant-velocity gradient elution. This strategy was validated through separations of mixtures of peptides, and we believe it will be effective and useful for a wide range of applications.

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