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Articles

Simultaneous Concentration and Separation of Coumarins Using a Molecular Micelle in Micellar Affinity Gradient Focusing

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We report the use of a molecular micelle for the simultaneous separation and concentration of neutral and hydrophobic analytes using micellar affinity gradient focusing (MAGF). The technique, MAGF, combines the favorable features of micellar electrokinetic chromatography and temperature gradient focusing. The focusing of neutral coumarin analytes was accomplished by the use the molecular micelle, poly(sodium undecenyl sulfate) (poly-SUS). Concentration enhancements of 10−25-fold/min were achieved for focusing of the coumarin dyes. The effect of varying the temperature gradient on the resolution of two of the coumarin dyes was also investigated, demonstrating that improved resolution could be achieved by reducing the steepness of the temperature gradient. In addition, with scanning-mode MAGF (in which the peaks are sequentially scanned past a fixed detection point by varying the buffer counterflow velocity), the use of poly-SUS was shown to produce repeatable and quantitative analyte peaks, making quantitative separations possible with the MAGF technique. Finally, it was shown that peak areas could be increased in scanning MAGF by reducing the scan rate so that the sensitivity of the method can be adjusted as needed.

Equilibrium gradient focusing techniques,[1] such as isoelectric focusing (IEF), combine high-resolution separation with built-in concentration enhancement. They are therefore potentially powerful analytical tools, particularly for low-concentration samples or in microfluidic systems where adequate detection limits may be difficult to achieve. However, their use is limited because there are only a small number of equilibrium gradient focusing techniques available. IEF is currently the only such technique that is widely used,[2−4] and while it has proven to be among the most powerful techniques for the analysis of proteins and peptides, it is limited to the separation of molecules with an isoelectric point between approximately 3 and 11. So, with few exceptions, it is restricted to the analysis of proteins and peptides.

Recently, a number of new equilibrium gradient focusing techniques have been described that are not limited to molecules with an isoelectric point and thus have the potential to be applied to a much broader range of analytes. Electric field gradient focusing (EFGF)[5−10] focuses ionic analytes by balancing their electrophoretic velocities against the bulk flow of solution through the separation channel. An arrangement of semipermeable membranes and electrodes is used to create an electric field that varies in strength along the length of the channel so that the total analyte velocity is equal to zero at a single point along the channel, and the analyte accumulates or is focused at that point. Different analytes, with different electrophoretic mobilities, will have different zero-velocity points, and so will be simultaneously concentrated and separated. Because of the use of semipermeable

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membranes, EFGF is typically limited to use with large molecules that will not pass through the membranes.

Temperature gradient focusing (TGF)[11] is similar to EFGF in that it balances electrophoresis against bulk solution flow. However, with TGF, the electrophoretic velocity gradient is produced using the combination of a temperature gradient along the separation channel and a buffer with a temperature-dependent ionic strength. Consequently, it can be used to focus both large and small analyte molecules. A variation of TGF that has been more recently described is scanning TGF[12] in which the bulk flow velocity is not held constant. It is instead varied over time, typically from high to low flow rates, over the course of a separation. In scanning mode, the zero-velocity points of the analytes are successively swept across the focusing gradient, and each analyte peak is detected at a fixed point near the end of the gradient.

Micellar affinity gradient focusing (MAGF)[13] also balances electrophoresis and bulk solution flow, but micelles are used to impart an electrophoretic mobility that depends on the strength of interaction between the analytes and the micelles. Furthermore, rather than using an electric field gradient or an ionic strength gradient to generate the electrophoretic velocity gradient (in which case, both the analytes and micelles would focus), MAGF works through the formation of a gradient in the interaction strength (retention factor) between the analytes and micelles. MAGF is thus a focusing-mode analog to micellar electrokinetic chromatography (MEKC) and can be used for the simultaneous concentration and separation of hydrophobic or neutral analytes, or both.

In principle, the retention factor gradient required for MAGF could be generated in a number of different ways, but perhaps the simplest was demonstrated in the first published paper on MAGF[13] and is used in this work as well. Briefly, a temperature gradient is produced along a separation channel containing an analyte/micellar system in which the retention factor is temperature dependent. The apparatus and mode of operation for MAGF are thus essentially the same as for TGF. Because MAGF uses micelles to impart an electrophoretic velocity gradient, however, it does not suffer from the same set of limitations as TGF. In particular, it is not restricted to use with buffers having a temperature-dependent ionic strength. When implemented with surfactant monomers, as in previous work,[13] however, MAGF is restricted to use with buffers that will support micelle formation. Furthermore, the use of a temperature gradient to form a controlled micellar retention factor gradient is complicated by the fact that the retention factor is the product of two terms: the distribution coefficient and the phase ratio. For surfactant monomer systems, both of these terms are typically temperature dependent: the distribution coefficient because of the temperature dependence of hydrophobic interactions, and the phase ratio because of the temperature dependence of the critical micelle concentration (cmc). These two contributions to the retention factor often trend in different directions with temperature leading to a total temperature dependence that is relatively weak or even nonmonotonic.

Molecular micelles,[14,15] also referred to as polymeric surfactants, have been used as suitable alternatives to conventional micelles as pseudostationary phases for MEKC separations due to their remarkable stability. The presence of covalent bonds, linking monomer molecules, eliminates the dynamic equilibrium that exists between monomer molecules and the normal micellar aggregate. Thus, unlike conventional micelles, molecular micelles do not have a cmc and can be used at very low concentrations. Furthermore, in contrast to conventional micelles where organic modifiers disrupt micelle formation, higher concentrations of organic modifier may be used without adversely affecting the properties of polymeric surfactants. Thus, the use of molecular micelles in lieu of conventional micelles is expected to be advantageous for MAGF since they can be used in a wider variety of buffers and at a wider range of concentrations without concern for the temperature dependence of the cmc.

In this paper, the use of the achiral molecular micelle poly(sodium undecenyl sulfate) (poly-SUS) in MAGF is investigated for the simultaneous focusing and separation of three coumarin dyes: coumarin 334 (C334), coumarin 450 (C450), and coumarin 460 (C460). The coumarin dyes were chosen because they are neutral, hydrophobic, and fluorescent. These properties allow us to examine nonionic species and their binding affinity to micelles and to image the focusing process with fluorescence microscopy. The results of this work are arranged into two sections: static MAGF with a fixed bulk flow rate, and scanning MAGF with a bulk flow rate that is scanned over the course of a separation as described above.

EXPERIMENTAL SECTION

Reagents and Chemicals. The coumarin laser dyes C460 and C450 were purchased from Exciton Inc (Dayton, OH), and C334 was purchased from Acros Organics (Geel, Belgium). The chemical structures of these analytes are shown in Figure 1. All reagents were used as received and prepared in ultrafiltered water from Fisher Scientific (Fair Lawn, NJ). Sodium phosphate dibasic, sodium borate, and sodium hydroxide were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol was obtained from Aldrich (Milwaukee, WI). The molecular micelle, poly-SUS, was synthesized according to the previously reported procedure.[14]

Buffer and Sample Preparation. For focusing using poly-SUS molecular micelles, the buffer solution consisted of 12.5 mmol/L Na_2HPO_4, 12.5 mmol/L $Na_2B_4O_7$, and 10% MeOH and was adjusted to pH 9.2 using 1 mol/L NaOH. The mobile phase for focusing experiments was prepared by dissolving poly-SUS in the buffer (0.125% w/v).

All solutions were sonicated and filtered using 0.2-μm polypropylene nylon filters before use. Stock solutions of the coumarin dyes were prepared by dissolving the dyes in pure methanol at a concentration of 500 μmol/L. The final analyte concentration was prepared by diluting with the appropriate amount of buffer.

Micellar Affinity Gradient Focusing Apparatus. Fluorescence Microscopy. Fluorescence microscopy experiments were performed using a Leica DM LB fluorescence microscope equipped with a 10× objective lens and a mercury arc lamp. The microscope filter set used for detection of the coumarin dyes consisted of a

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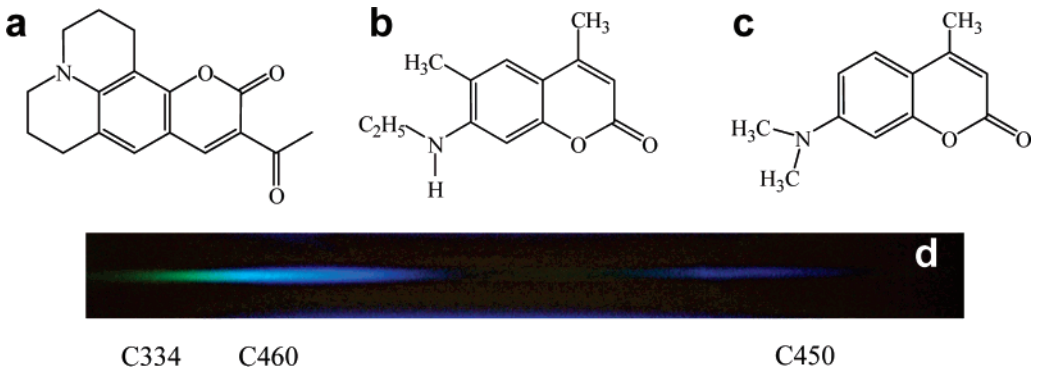

Figure 1. Structures of coumarin dyes investigated: (a) C334, (b) C460, and (c) C450. (d) Fluorescence micrograph illustrating the focusing and separation of C334 (green), C460 (blue), and C450 (blue). Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mmol/L $Na_2B_4O_7$, and 12.5 mmol/L Na_2HPO_4 at pH 9.2; voltage, −2000 V (voltage applied to sample reservoir); temperature gradient 35 °C/mm (T_H = 80 °C (left side of figure) and T_C = 10 °C (right side of figure); capillary, 3 cm × 30 μm i.d; gradient zone, 2 mm. Initial analyte concentration, 25 nmol/L (each coumarin). For scale, the image is 2 mm long.

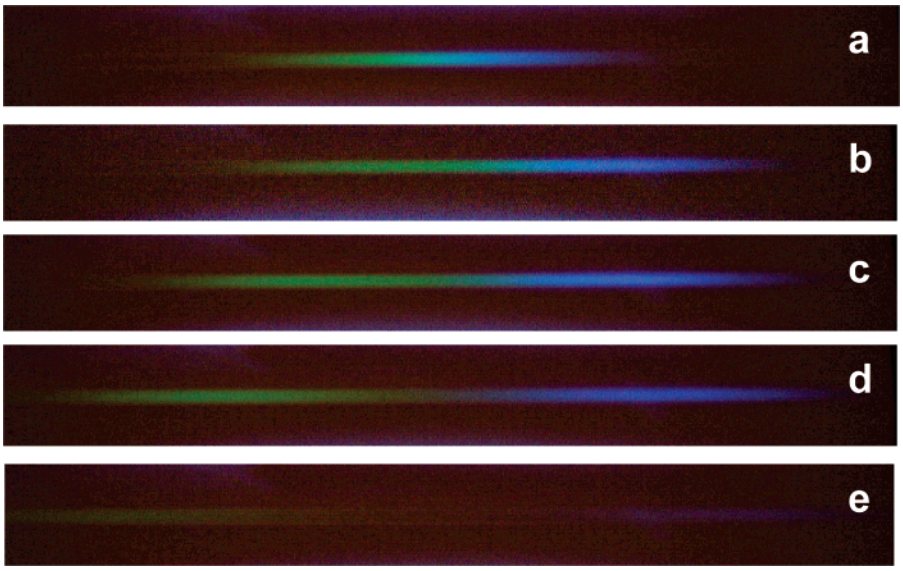

Figure 2. Fluorescence micrographs illustrating the effect of varying temperature gradient on resolution of C334 (green) and C460 (blue). Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mmol/L $Na_2B_4O_7$, and 12.5 mmol/L Na_2HPO_4 at pH 9.2; voltage, −2000 V (voltage applied to sample reservoir); temperature gradient: (a) 35 °C/mm (T_H = 80 °C, T_C = 10 °C); (b) 25 °C/mm (T_H = 60 °C, T_C = 10 °C); (c) 20 °C/mm (T_H = 50 °C, T_C = 10 °C); (d) 15 °C/mm (T_H = 40 °C, T_C = 10 °C; (e) 10 °C/mm (T_H = 30 °C, T_C = 10 °C). For scale, each image is 2 mm long.

350 ± 25 nm band-pass excitation filter and 420-nm long pass emission filter. All digital images were acquired using a color CCD camera (Dage-MTI22) using Scion Image software and a Scion CG-7 frame grabber (Scion, Inc., Frederick, MD).

Capillary Device Preparation. Separation of the coumarin dyes was performed using a capillary device consisting of a 3-cm-long fused-silica capillary (30-μm i.d., 360-μm o.d.) purchased from Polymicro Technologies, LLC (Phoenix, AZ). A 5-mm optical window on the silica capillary was prepared by burning a portion of the outer polyimide coating. The capillary was then embedded between two polycarbonate sheets (McMaster Carr, Atlanta, GA) by inserting the capillary between the sheets, placing the sheet/capillary/sheet assembly in a hydraulic press for 5 min at 180 °C, 4500 N, and cooling to 120 °C before releasing the pressure. To prevent crushing of the capillary and to define the final thickness of the assembled device, two metal shims (500 μm thick) were placed in the hydraulic press beside the assembly prior to application of the pressure and temperature.

The apparatus for applying the temperature gradient and controlling the bulk flow has been described previously.[12,16] Briefly, the sample reservoir and the input end of the capillary were anchored to a copper block connected to a recirculating water bath and maintained at a relatively low temperature (T_C). A section of the middle of the capillary (~1 cm from the input end and 0.6 cm long) was regulated at a relatively high temperature (T_H) by anchoring it to a copper block with an embedded thermoelectric heater. The gap between the two blocks defined the temperature gradient zone and was set to 2 mm. The capillary was connected at the input end to a polypropylene sample reservoir (150-μL volume) via a 360-μm hole drilled into the reservoir, and at the output end to the waste reservoir via a PTFE-backed silicone rubber septum. The waste reservoir was sealed, and the pressure in the waste reservoir was precisely controlled either using a

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precision pneumatic pressure controller or a gravity-fed siphon on a precision computer-controlled vertical translation stage.

Before each separation, the capillary was filled with the mobile phase and the sample reservoir was filled with the analyte solution. A high voltage was then applied to the sample reservoir while the waste reservoir was electrically grounded to simultaneously separate and concentrate the analytes. During the separation, the analytes were continuously injected into the capillary.

RESULTS AND DISCUSSION

Static MAGF. The molecular structures of the three coumarin dyes used for this study are shown in Figure 1a−c along with a fluorescence micrograph (Figure 1d) of the focusing capillary during focusing of all three dyes: C334 (green), C460 (blue), and C450 (blue). The mobile phase used consisted of 0.125% w/v poly-SUS in a buffer composed of 12.5 mmol/L $Na_2B_4O_7$ and 12.5 mmol/L Na_2HPO_4 at pH 9.2. Higher poly-SUS concentrations resulted in the analyte precipitating with the molecular micelle, which often led to capillary blockage.

At a temperature gradient of 35 °C/mm (T_H = 80 °C and T_C = 10 °C), C460 and C450 dyes were completely resolved; however, C334 and C460 partially overlapped at their focus points (Figure 1d). Figure 2 illustrates how the resolution of C334 and C460 was improved by reducing the steepness (slope) of the temperature gradient. The steepness of the temperature gradient was varied in steps by adjusting T_H of the microchannel from 80 to 30 °C while T_C and the temperature gradient length were held constant at 10 °C and 2 mm, respectively. Once the desired temperature gradient was reached, a mixture of the two coumarin dyes was injected and a voltage was applied to achieve focusing. Each image of Figure 2 was taken after ~2 min of focusing. As shown in the figure, the resolution of the two coumarin analytes increased as the steepness of the temperature gradient was decreased. The use of a lower temperature gradient is equivalent to the use of longer capillaries in CE, where better resolution is achieved with a longer migration time.

An advantage of MAGF is the ability to achieve concentration enhancement with an increase in focusing time while maintaining peak resolution. The concentration enhancement of C334 and C460 as a function of time was investigated. Figure 3a−f shows fluorescence micrographs of the concentration enhancement of the C334 observed every 2 min during focusing. The initial concentration of C334 in this experiment was 25 nmol/L with a final concentration of 2500 nmol/L corresponding to a 100-fold enhancement in 10 min. Figure 3g is a plot of the peak analyte concentration versus time for a similar experiment with C460 as the analyte. The nonlinearity of the curve—with a slower apparent focusing rate at later times—is not well understood. Possible explanations of the nonlinearity are photobleaching, inner filter effects, dye self quenching, the onset of a saturation of the micelle-mediated focusing, or a combination of more than one of these effects. In this experiment, the initial analyte concentration of C460 was 5 nmol/L, and after focusing for 10 min, the final concentration was 1.3 μmol/L, corresponding to a 260-fold concentration enhancement. The concentration values in Figure 3 were determined from calibration plots obtained by measuring the fluorescence of various concentrations of C334 and C460 in the capillary device under identical temperature conditions but with no voltage applied.

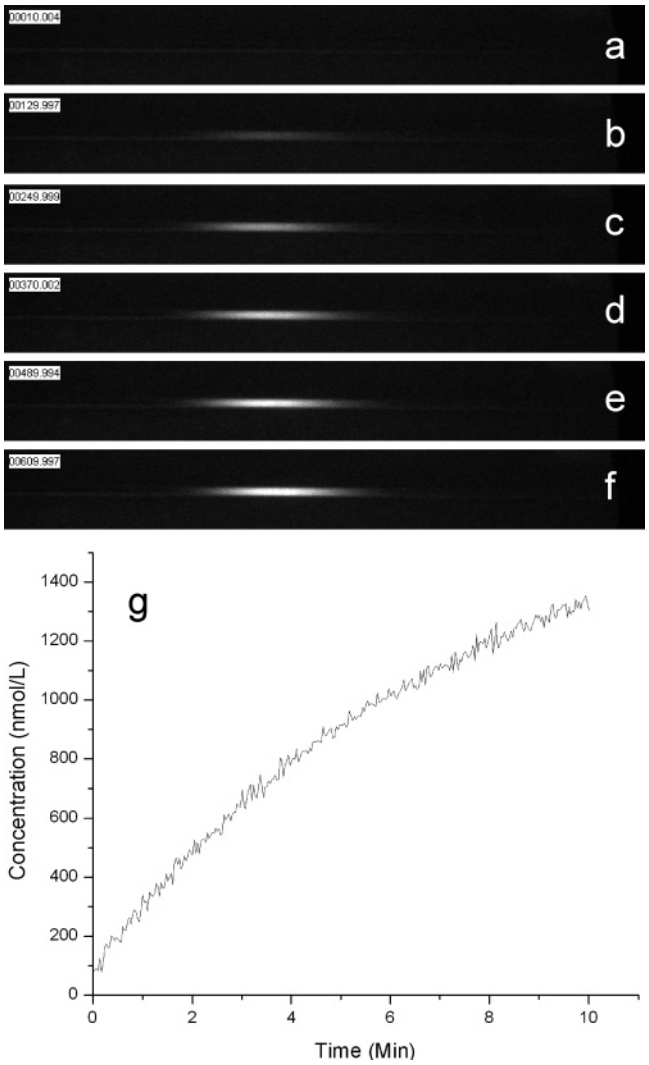

Figure 3. (a−f) Fluorescence micrographs illustrating the focusing of C334 as a function of time with poly-SUS micelles. Images were taken in 2-min intervals after voltage application. Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mmol/L $Na_2B_4O_7$, and12.5 mmol/L Na_2HPO_4 at pH 9.2; voltage, −2000 V (voltage applied to sample reservoir); temperature gradient, 35 °C/mm (T_H = 80 °C, T_C = 10 °C). Initial analyte concentration, 25 nmol/L; analyte concentration after 10 min, 2.5 μmol/L. (g) Plot of concentration as a function of time for C460. Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mmol/L $Na_2B_4O_7$, and 12.5 mmol/L Na_2HPO_4 at pH 9.2; voltage, −1000 V (voltage applied to sample reservoir); temperature gradient, 35 °C/mm (T_H = 80 °C, T_C = 10 °C). Initial analyte concentration, 5 nmol/L; analyte concentration after 10 min, 1.3 μmol/L. For scale, each image is 2 mm long. The time stamp at the upper left of each image is in seconds. The voltage was turned on at the same time as the capture of image a.

Quantitative Focusing with Scanning-Mode MAGF. One of the disadvantages of the static MAGF method presented above is that the rate of analyte focusing is dependent on the location of the focusing point along the gradient zone. Note that the intensity of the C450 peak is much less than that of the C334 and C460 peaks in Figure 1d even though the initial sample concentration of the three coumarins was the same. This would make quantitative analysis difficult since any small variation in the bulk flow rate—such as from variations of the shape of the meniscus in the sample reservoir—would result in a variation not only of

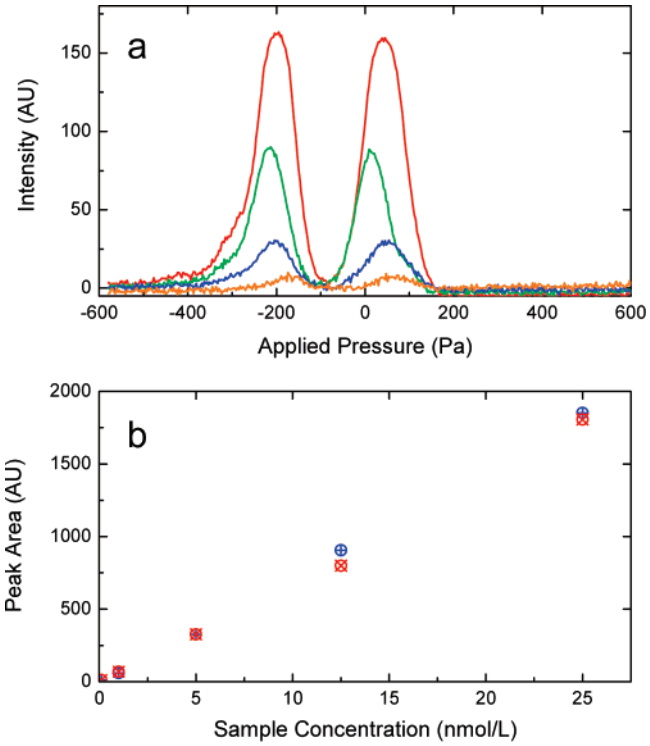

Figure 4. Scanning MAGF of C450 (left peak) and C460 (right peak). Effect of varying initial sample concentration on (a) Peak Intensity (b) Peak areas for C450 (blue ⊕) and C460 (red ⊗). Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mmol/L $Na_2B_4O_7$, and 12.5 mmol/L Na_2HPO_4 at pH 9.2; voltage, −1000 V (voltage applied to sample reservoir); temperature gradient, 35 °C/mm (T_H = 80 °C, T_C = 10 °C); scan rate 77 Pa/min; sample concentrations (from top to bottom of (a)): 25 (red), 12.5 (green), 5 (blue), and 1 nmol/L (orange). Note that when plotted as a function of pressure, the peak order is reversed relative to the micrographs of Figures 1 and 2.

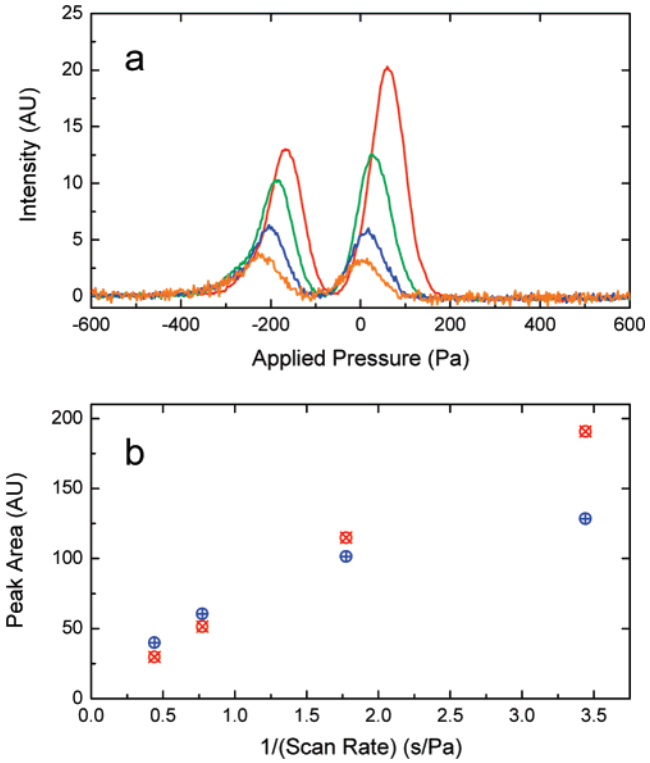

Figure 5. Scanning MAGF of C450 (left peak) and C460 (right peak). Effect of changing scan rate on (a) peak intensity (b) peak areas at 1 nmol/L initial sample for C450 (blue ⊕) and C460 (red ⊗). Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mmol/L $Na_2B_4O_7$, and 12.5 mmol/L Na_2HPO_4 at pH 9.2; voltage, −1000 V (voltage applied to sample reservoir); temperature gradient, 35 °C/mm (T_H = 80 °C, T_C = 10 °C). Scan rate: (from top to bottom of (a)): 17 (red), 34 (green), 77 (blue), and 136 Pa/min (orange). Note that when plotted as a function of pressure, the peak order is reversed relative to the micrographs of Figures 1 and 2.

the peak position but also of the peak height and area. A modification to the method that has been shown to be effective in providing quantitative peak areas with TGF separations is to vary or "scan" the bulk flow rate over the course of a separation.[12] With scanning MAGF, peaks are sequentially focused and eluted past a fixed detection point. Consequently, the degree that each analyte is focused before reaching the detection spot is approximately the same for each analyte (assuming a constant rate of change of the bulk flow rate). In a typical MAGF experiment, the bulk flow is primarily driven by electroosmosis, but in order to control the bulk flow velocity, a precisely controlled pressure is applied to the waste end of the capillary as described above. During a scanning MAGF separation, the applied pressure is varied from high to low and the fluorescence intensity is monitored at a fixed point along the gradient zone (for the examples of Figures 4 and 5 the detection spot was ~80 μm long, located ~450 μm from the hot edge of the temperature gradient zone). The resulting plot of fluorescence intensity versus applied pressure is then roughly equivalent to a conventional electropherogram.

Figure 4a shows the resulting plots for a series of scanning MAGF separations of C450 and C460 at various concentrations. In each case, the peaks for C450 and C460 are very similar in height and width. The peak positions are also found to be quite reproducible—with some small variation (smaller than the peak widths) due to variations in sample volume and sample meniscus shape. A plot of peak area versus initial sample concentration is shown in Figure 4b. As expected, an increase in analyte concentration resulted in an increase in peak area, with both analytes showing a linear response (R^2 = 0.999 and 0.996 for C450 and C460, respectively) over the concentration range used.

Because MAGF is a focusing method, the area of an analyte peak depends not only on the initial sample concentration but also on the time over which the analyte is injected and focused. With the scanning MAGF method, this allows the peak areas (and therefore detection limits) to be varied as needed simply by changing the rate at which the applied pressure is scanned. Figure 5a shows a series of intensity versus applied pressure plots for scanning MAGF separations of C450 and C460 at various scan rates. Because the bulk flow rate is scanned over time rather than left constant, the zero-velocity point for each analyte moves along the temperature gradient. Consequently, the focused analyte peaks also move along the temperature gradient, but at a small distance behind their zero velocity points. At faster scan rates, the distance between the focused peak and the zero-velocity point is larger. Consequently, there is a systematic variation of peak position with scan rate in Figure 5a. Figure 5b shows the resulting peak areas plotted as a function of the inverse scan rate. Because the focusing time for each analyte is proportional to the inverse scan rate, it

would be expected that the peak area plots of Figure 5b would be linear. Although they appear to be fairly linear for fast scan rates, at slower scan rates, the peak areas are significantly reduced from what would be expected, particularly for C450 (the left peak in Figure 5a). As with the nonlinearity of Figure 3g, this apparent reduction in focusing rate could be due to a number of factors including photobleaching, inner filter effects, dye self-quenching, and the onset of a saturation of the micelle-mediated focusing. A similar nonlinearity was found for scanning TGF of fluorescein dyes,[12] and in that case, it was shown to be due to photobleaching.

CONCLUSION

MAGF, a technique for simultaneous separation and concentration of hydrophobic analytes has been demonstrated using a molecular micelle. The use of molecular micelles in lieu of conventional monomer surfactants has two primary advantages: (1) it eliminates the requirement for a temperature-dependent cmc, so that a larger range of mobile-phase compositions can be used; and (2) a variety of different molecular micelles—with different selectivities—are available .[17,18] Consequently, the combination of MAGF with molecular micelles has the potential to greatly expand the range of applicability of equilibrium gradient focusing techniques.

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