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Research Article

Electrophoretic exclusion for the selective transport of small molecules

A novel method capable of differentiating and concentrating small molecules in bulk solution termed ''electrophoretic exclusion'' is described and experimentally investigated. In this technique, the hydrodynamic flow of the system is countered by the electrophoretic velocity to prevent a species from entering into the channel. The separation can be controlled by changing the flow rate or applied electric field in order to exclude certain species selectively while allowing others to pass through the capillary. Proof of principle studies employed a flow injection regime of the method and examined the exclusion of Methyl Violet dye in the presence of a neutral species. Methyl Violet was concentrated almost 40 times the background concentration in 30 s using 6 kV. Additionally, a threshold voltage necessary for exclusion was determined. The establishment of a threshold voltage enabled the differentiation of two similar cationic species: Methyl Green and Neutral Red.

Keywords:
Electrophoretic Exclusion / Counterflow / Electrocapture / Gradient Separations
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1 Introduction

The field of separations science has made significant contributions towards biological analysis. One separations technique, capillary electrophoresis (CE), has been used for numerous biochemical applications [1–8]. However, one of the major disadvantages of CE is its poor concentration limits. To enhance CE, several methods of sample preconcentration have been investigated. Some common approaches include field-amplified sample stacking [9–11], transient isotachophoresis [12–15], moving reaction boundary [16–18], and dynamic pH junction [19–21], among others. Another approach to improving electrophoretic separations is through the use of equilibrium gradients. Giddings and Dahlgreen introduced the term ''equilibrium gradients'' in 1971 [22]; in these techniques, there exists an imbalance in the net force that the analyte experiences within the channel. The species will migrate through the capillary until the forces that it encounters are equal, and the analyte movement then comes to rest at that point in the separation channel. Analytes with dissimilar properties will come to rest at unique zero velocity points in the capillary. A type of equilibrium gradient technique is electrofocusing or electrocapture using counterflow. In this regime, electrophoresis is countered by the bulk solution flow, which can be applied through external pressure, hydrodynamic force, or electroosmotic force [23–32]. A species will then come to rest where its electrophoretic velocity is equal to the counterflow [33].

The technique introduced in this paper is termed ''electrophoretic exclusion'' and is the result of two opposing forces: hydrodynamic flow and electrophoretic velocity. It utilizes similar principles of counterflow separations in which the analytes are separated by counteracting forces; however, in this method, the forces oppose one another at the entrance of a capillary rather than within a capillary or chamber. Whereas most other equilibrium gradient techniques focus species within a channel, this method accumulates analytes in the bulk solution. A few other counterflow methods have excluded species from a capillary, but these are multi-step processes that also utilize separations or enhancement within the capillary [34, 35]. When the electrophoretic velocity of a species is greater than the hydrodynamic flow into the capillary, the species is excluded from the channel. As long as electric field is being applied, the analyte will continue to be excluded and concentrate. A method with similar principles was published by Hori *et al.*, but the technique employed large volumes and a 1.5 mm diameter tube [36]. Initial work by Polson *et al.* utilized this electrophoretic exclusion technique and demonstrated the ability to exclude and concentrate 200 nm particles at the entrance of a 20 μm i.d. capillary [37]. Furthermore, differential transport at the capillary entrance was later modeled by Pacheco *et al.* to investigate the various conditions that impact exclusion [38].

In this work, we present a flow injection device that utilizes the principles of electrophoretic exclusion to concentrate and differentiate small dye molecules. This

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electrophoretic exclusion device has the potential to be extended to contain multiple ''gates,'' or exclusion points, either in series and/or in parallel for rapid analysis of complex fluids. Demonstration of successful exclusion of a species at one gate, which is the subject of this work, is a critical step before more intricate devices can be developed. In order to characterize the system and demonstrate device functionality, small dye molecules were examined. The method establishes an ability to exclude and concentrate Methyl Violet dye while allowing a neutral dye to move through the channel. Additionally, two similar cationic species were able to be separated based upon their different electrophoretic mobilities.

2 Materials and methods

2.1 Reagents

DL aspartic acid, Methyl Violet, (MW = 393.9, Sigma Aldrich, St. Louis, MO), hydrochloric acid (HCl, Mallinckrodt, Hazelwood, MO), Neutral Red (MW = 288.8, Mallinckrodt Baker, Phillipsburg, NJ), Methyl Green (MW = 458.5, Allied Chemical Corp., New York, NY), and Martius Yellow (MW = 234.2, Eastman Organic, Rochester, NY) were used as received. Aspartic acid buffer was prepared to 5 mM concentration using 18 MΩ water and adjusted to pH 2.85 using 1 M HCl. Dye solutions were prepared to 1 mM with the 5 mM aspartic acid buffer and diluted to 50 μM for individual trials.

2.2 Instrumentation

Polyimide-coated fused silica capillaries (13 cm in length, 75 μm i.d. 150 μm o.d., Polymicro Technologies, Phoenix, AZ) were used to connect two modified 2 mL glass vials. A window was burned mid-way on the capillary (~6 cm) to allow for absorbance detection. One end of the capillary was sputter-coated with 30 nm titanium and then 50 nm platinum after removing a small portion (~0.5 cm) of the polyimide coating. The sputter-coated tips were physically connected to a platinum wire using silver conducting epoxy. All surfaces except for the very tip of the capillary and~1 mm at the end of the platinum wire were coated with epoxy so that only the capillary tip was conducting and that a flat potential field existed in the reservoir [37]. This design allows the potential field to be initiated immediately at the capillary entrance.

The vials and capillaries were mounted on a rotatable board (not shown) so that the flow rate could be controlled by gravity pressure (Fig. 1). The glass vials were modified so that they were open to air and dye could be added as necessary with a syringe. For the experimental trials presented in this paper, the height between the menisci in the vials was 1.5 cm, and the calculated flow rate was 0.88 nL/s.

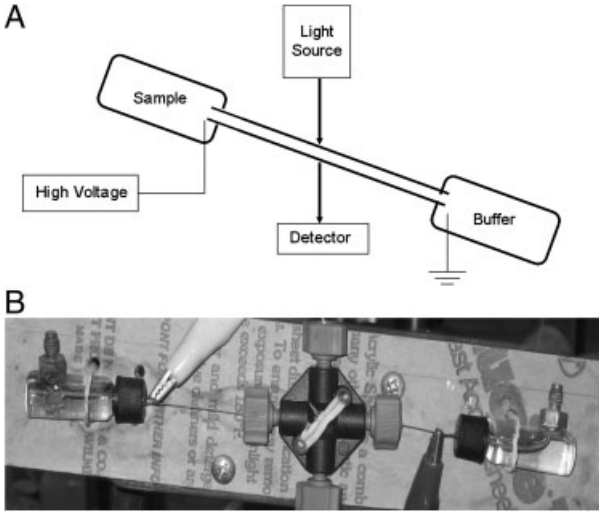

Figure 1. Instrumentation used for the exclusion technique. Fig. 1A is schematic of the setup, while Fig. 1B is a photographic image. A 13 cm capillary (75 μm i.d.) connects two modified reservoirs, and hydrodynamic flow travels from the sample vial (on the left) to the buffer vial (on the right). Detection is performed 6 cm from the entrance of the capillary in the sample vial using absorbance. Potential is applied at the entrance of the capillary, while the potential field in the reservoir remains flat.

The electrophoretic exclusion system was built in-house and used a CZE1000R high voltage power supply (Spellman High Voltage Electronics Corporation, Hauppauge, NY), a Mikropack DH-2000 UV-vis light source, CUV CCE Electrophoresis sample cell, and a USB2000 Spectrometer (all Ocean Optics, Dunedin, FL). OOIBase software (Ocean Optics, Dunedin, FL) was used for data collection. The absorbance intensity was monitored mid-capillary.

3 Results and discussion

3.1 Principles of electrophoretic exclusion

The electrophoretic exclusion method utilizes the principles of counterflow techniques to prevent certain classes of species from entering the capillary, creating differential behavior that can be exploited for separation and concentration. In this contribution, the entrance interfacial areas are being investigated by a flow injection-like strategy. The critical parameters necessary for exclusion include the electrophoretic mobility of the analyte of interest, the hydrodynamic flow, and the applied electric field. Exclusion occurs when the average electrophoretic velocity is greater than or equal to the average hydrodynamic flow velocity [38]. Because the electrophoretic mobility is constant for a set of experiments, defined by the buffer and the target species, the exclusion can be controlled by varying flow and electric fields.

In order to differentiate two species under a set flow field, an electric field must be chosen that is high enough to

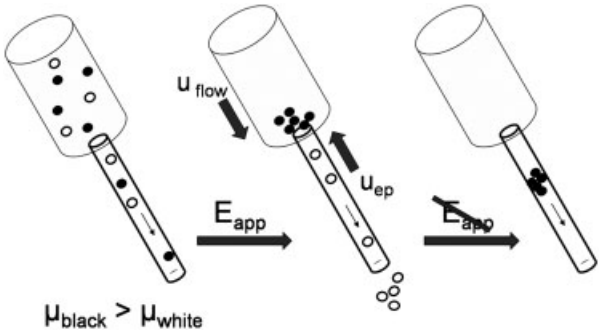

Figure 2. Schematic of the electrophoretic exclusion effect. The left vial represents the system with no electric field present. All species in the vial flow from the vial through the capillary with the hydrodynamic flow. The middle vial demonstrates the exclusion effect when the potential is applied. Because the dark circles have a larger electrophoretic mobility, their movement is arrested at the entrance of the capillary, whereas the lighter species is relatively unaffected by the introduced force. The vial on the right shows the release of the collected species once the potential is removed.

exclude one species while allowing a lower mobility species to pass through the capillary (Fig. 2). In Fig. 2, the black species has a higher electrophoretic mobility than the white species. The system on the left is influenced by only hydrodynamic flow; that is, gravity-induced flow carries the buffer and both species from the vial through the capillary. When an electric field is applied within the capillary that is high enough to exclude the black species but not the white, then the black species is excluded from the capillary, while the movement of the white species remains relatively unaffected by the field (Fig. 2, middle). The darker species continues to move with the hydrodynamic flow towards the entrance of the capillary; however, once the species encounters the electric field at the capillary entrance, the forces oppose one another and the movement is retarded or reversed. This is similar to the zero velocity point in equilibrium gradient focusing techniques. In contrast to other focusing or electrocapture techniques, species are differentiated in the bulk solution at the interface of the opposing forces outside of the capillary instead of focusing them in a band within the capillary. As long as the electric field is applied, the species will continue to be excluded. Once the electric field is removed, the excluded material can enter into the capillary with the hydrodynamic flow (Fig. 2, vial on right).

3.2 Proof of principle experiments

Experiments were performed with various dyes to establish functionality and characterize the system. The initial proof of principle experiments studied cationic Methyl Violet dye ($\lambda_{max} = 580$ nm, MW = 393.9, $\mu_{ep} = 1.7 \times 10^{-4}\,cm^2\,V^{-1}s^{-1}$) and neutral Martius Yellow dye ($\lambda_{max} = 435$ nm, MW = 234.2,) to demonstrate electrophoretic exclusion (Fig. 3). The peak on the signal trace represents the cationic dye molecules that were excluded from the capillary and concentrated. Using the method, Methyl Violet was excluded and concentrated while the neutral dye was unaffected by the applied field. Because the detector is set up mid-way down the capillary, it takes over 200 s before the collected material reaches the detector once the potential is removed. A third wavelength (675 nm) was monitored as an internal control in all experiments to ensure that there were no physical disturbances of the instrument that would cause a fluctuation in signal (shown in Fig. 3 inset). All three wavelengths were monitored and recorded simultaneously.

Various controls were performed to ensure that the peaks generated were a result of the countering of hydrodynamic flow and electrophoretic velocity and not from other phenomenon (Fig. 3, inset). In order to confirm that no peaks would form without the necessary forces, control trials were performed similarly to a standard experiment, but without one significant parameter (flow, electric field, or charged dye). In the experiments without flow, the rotatable board was adjusted so that there was no change in height between the menisci in the vials, causing the fluid to be stagnant. For the next control, the board was tilted to achieve the experimental flow rate, but no voltage was applied. Lastly, a trial was performed by applying electric field to only buffer as it flowed through the system. The traces collected for each of these trials showed neither signal change nor concentration increase (Fig. 3, inset). Control trials were monitored longer than an experimental trial to confirm that there were no delayed effects. These controls both confirmed the principles supporting the electrophoretic exclusion of the necessary juxtaposition of forces, as well as ensured that no outside force was generating a peak in the signal traces. The experimental trials were also very

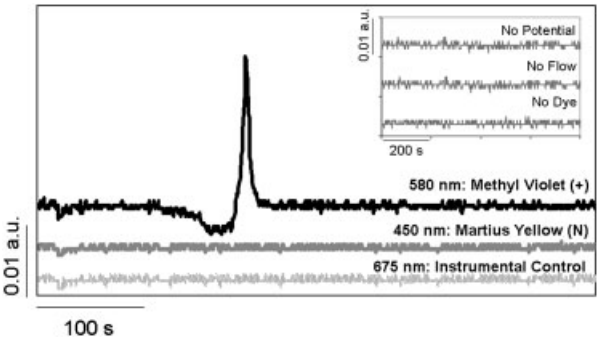

Figure 3. Demonstration of exclusion using cationic Methyl Violet dye. Three wavelengths were monitored simultaneously. The black line, gray line, and lighter gray line represent Methyl Violet cationic dye (580 nm), an instrumental control (675 nm), and Martius Yellow neutral dye (435 nm), respectively. These traces are offset for clarity. Both the neutral dye and the instrumental control (for detecting physical fluid disturbances) showed no response, while the Methyl Violet trace indicates an increase in concentration. The inset shows the three control trials and the traces are labeled accordingly to which experimental parameter was removed. The control traces also do not display any response.

reproducible in nature; changing buffers or capillaries did not have any obvious effects on the results.

Additional experiments were performed to study the ability to manipulate the collected material. A micro-stir bar was added to the upper vial so that the excluded material could be disturbed prior to entering into the capillary. Immediately after the potential was removed, the solution was agitated with the stir bar for approximately 20 seconds. The resulting electropherograms were similar to the controls in that no concentration increase was observed. It was calculated that the system can detect concentration changes as low as 0.3 μmol/L, thus if any Methyl Violet was concentrated, it was less than this detection limit. These experiments have two important interpretations: first, the collected material is forming out in solution, away from the capillary tip, and second, the concentrated material can be manipulated in solution. The bulk solution, presumably, has become slightly enriched with the excluded dye, but this would be an undetectable difference with the current experimental regime.

An interesting trend of this technique is the time that it takes for a peak to be observed on the signal trace. Although all peaks appear after 200 s as the flow rate dictates, and peaks are always observed when the necessary forces are present, the location of the peak often varies, most frequently between 200 s and 400 s. This disparity in time suggests that once the bolus has formed, the convective currents in the vial are influencing it before it enters into the capillary. Because of the repeatability of the peak in trials, especially in terms of peak size or dye concentrated, the shifting of the peak is being treated as further supporting evidence that the bolus forms in bulk solution.

In order to examine the capability of the technique as a concentration device, the time of the applied potential was varied to determine if the amount of Methyl Violet continued to increase in the area near the capillary entrance. In 30 s, the Methyl Violet dye was concentrated approximately 40 times as high as the initial bulk concentration with 6 kV and a current of 12 μA (Fig. 4). The concentration enhancement was determined by dividing the amount of material collected (μg) by the background amount of dye (μg). The technique exhibited a nearly linear relationship between the length of time that the potential was applied and the amount of material collected. The trendline intercepts zero as the control experiments demonstrated that no dye is collected if no potential is applied. These results suggest that, within limits, material will continue to be excluded and concentrated for as long as the electric field is applied. Additionally, although the enhancement appears to be modest, it is relatively comparable to other counterflow electrofocusing techniques, especially as it is only examining the local bolus that is under significant hemi-spherical diffusional forces. In one temperature gradient focusing technique, one minute of focusing resulted in a 40-fold concentration enhancement [39], while a micellar affinity gradient focusing technique provided 27-fold enhancement in 30 s [40]. Furthermore, the original work in electric field gradient focusing provided a mere 2–3x enhancement in hours [26], whereas more recent developments in the area have demonstrated up to 4000-fold enhancement in 60 min [41]. Therefore, despite modest enhancement in the initial experiments, the method has the potential to be modified into a significant concentrating technique when using a constrained sample volume or employing these exclusion principles in series.

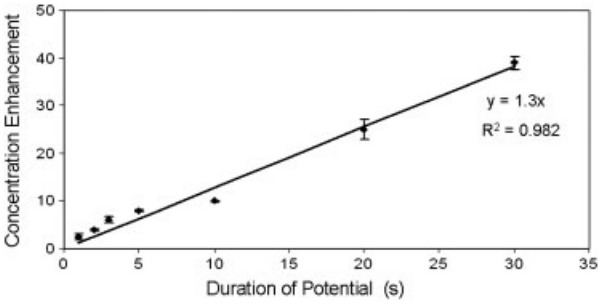

Figure 4. Average concentration enhancement of Methyl Violet. The concentration enhancement was calculated by dividing the amount of material collected with the technique by the background. Each point represents three trials.

3.3 Potential limitations

Because EOF complicates the flow and the rate is critical to this technique, the EOF was minimized by using a buffer at pH 2.85 [42–44]. Additionally, this pH is at the pI of the zwitterionic aspartic acid buffer employed, so the buffer ions have no net charge, and there are reduced counterions in solution which minimizes complications arising from buffer ions. In future experiments with different analytes such as proteins, buffers at physiological pH will be employed, and different capillary coatings will be investigated to limit the EOF [45–49].

In addition, Joule heating also has the potential of impacting this technique. Changes in temperature would alter both the viscosity of buffer and the electrophoretic mobility of the analytes as well as impact the diffusion rates in the system. The present data indicate that Joule heating is not influencing our current experimental regime.

Furthermore, electrolysis is undoubtedly occurring at the conductive surfaces surrounding the entrance and within the bulk sample. The overall current under our experimental conditions is approximately ten microamperes. The exact distribution of the current across the conductive surfaces is unknown, but clearly some of the resulting reactions will potentially influence the local environment about the entrance. The reaction at the entrance to capillary under these conditions is largely and most likely $2H^+ + 2e^- \rightarrow H_2(g)$, and the solubility of hydrogen gas in water is approximately 1 mM. Even if it were assumed

that the entire reservoir volume (2 mL) is available for hydrogen distribution, for saturation to occur, approximately 10^{-6} mole of hydrogen would need to be generated. At ten microamperes, this is several minutes. If the hydrogen is diffusion limited, then we can examine a diffusional hemisphere after one second which occupies a few nanoliters and has a concentration somewhat above saturation. However, this volume is swept by the convective flow of about a nL/s, so that local concentration is effectively minimized by convective flows. In fact, bubbles have not been a consistent experimental difficulty.

3.4 Analysis of the signal decrease before peak formation

What is also noteworthy is that a signal decrease occurs in all trials just prior to the appearance of the peak (Fig. 3, '580 nm: Methyl Violet (+)' data set). This dip in signal is observed in all data sets, yet none of the controls. The size of the dip remains relatively fixed with increasing applied fields, while the amount of material collected steadily increases (Fig. 5). With longer applications of potential (greater than 150 s), the dip migrates to appear significantly earlier than the peak (up to 100 s earlier when the potential was applied for 450 s). From this data, the distance from the dip to the detector was determined at the time that the potential was removed (Fig. 6). Not only does a linear relationship exist between the duration of applied potential and the distance to the detector, but also the dip comes within 2 cm of the detector within the capillary while the potential is being applied and the Methyl Violet is excluded. The behavior of this dip phenomenon appears to be similar to capillary electrophoresis migrating vacancy system peaks [50–53]. Although the dip is an interesting occurrence, because of its consistent and controlled nature and its similarity to the vacancy peaks, further investigation is held for future studies.

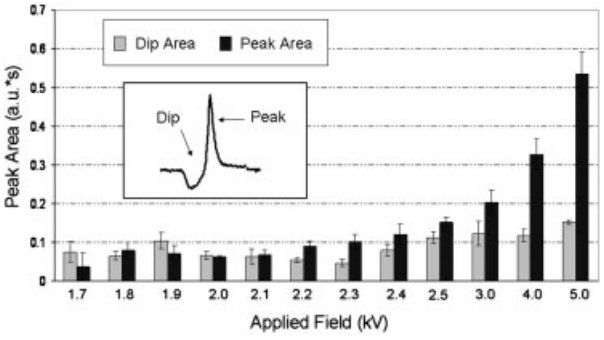

Figure 5. Stability of the area of the signal decrease compared to the increase in peak area for increasing applied field. The potential was applied in increments from 1.7 kV to 5.0 kV and both the peak area and area of the signal decrease (both labeled on the inset) were recorded. The error bars represent three experimental trials.

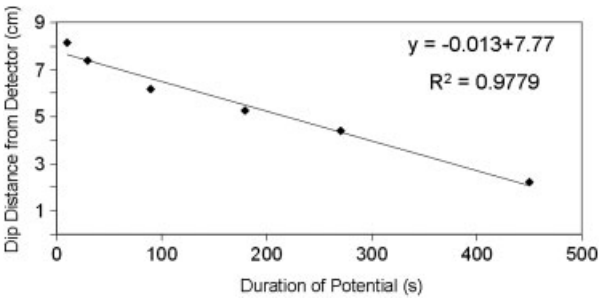

Figure 6. Calculated distance from the decrease in signal to the detector. The longer the duration that the potential is applied, the shorter the time it takes for the decrease to be observed, and thus the closer that it is to the detector. Three experimental trials are signified by the error bars.

3.5 Differentiation of two cationic species

The proof of principle experiments using Methyl Violet and control experiments demonstrate the basic premise of the strategy to manipulate small dye molecules; however, to create parallel or serial systems, very similar species would need to be differentially transported at the interface. Models suggest that a threshold voltage exists depending on the system's flow rate, applied field, and electrophoretic mobility [38]. Thus, if the flow rate and applied field remain constant, a threshold voltage should exist based upon the electrophoretic mobility of a species. To verify the existence of a threshold, the applied electric field was varied (Fig. 7). In these trials, voltages from 1.5 to 6.0 kV were applied for 5 s increments. The data indicates that an applied potential less than 1.7 kV is insufficient to exclude species from entering into the capillary intimating a threshold voltage necessary for exclusion. These results correlate well with the modeling performed by Pacheco *et al.* that proposes a certain threshold voltage exists for complete exclusion [38]. The exclusion is theorized to occur at 1.5 kV using the parameters in these experiments and trapping occurs with potentials as low as 1.7 kV, thus the model and experimental results are reasonably close.

The establishment of a threshold voltage has important ramifications: each analyte has a threshold voltage dependent on its electrophoretic mobility; thus, under the same experimental conditions, different analytes with individual electrophoretic mobilities will be excluded at unique potentials. To further examine this concept, two cationic species, Methyl Green (MW = 458.5, $\mu_{ep} = 1.3 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$) and Neutral Red (MW = 288.8, $\mu_{ep} = 3.1 \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$), were studied (Fig. 8). These dyes were monitored at 635 nm and 520 nm, respectively, each in a region where there is no overlap in their absorption spectra. In these trials, the potential was applied for 5 s but varied in intensity. The results indicate that Neutral Red dye was able to be excluded at potentials as low as 2.0 kV, whereas the Methyl Green dye was unable to be excluded until 3.0 kV. These results suggest that the

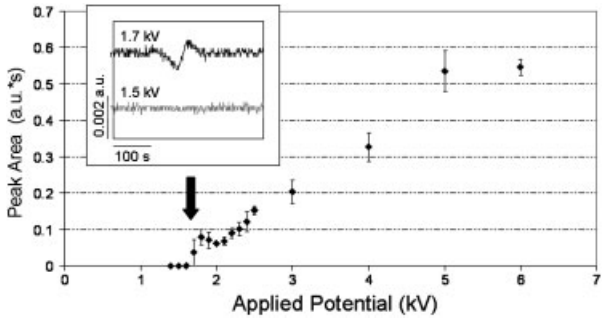

Figure 7. Determination of a threshold voltage for exclusion. Different magnitudes of potential were applied for 5 s for three trials. No concentration effect was observed for any potential under 1.7 kV. The inset shows the experimental traces for both 1.5 and 1.7 kV.

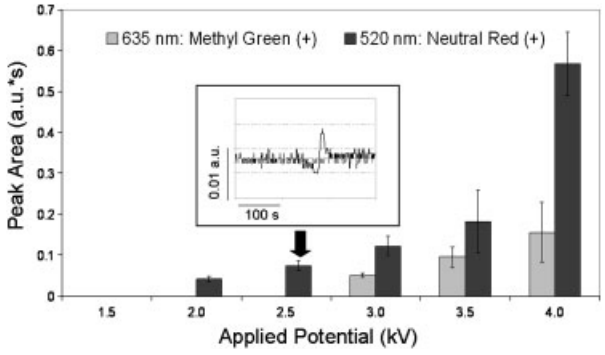

Figure 8. Collection of Methyl Green and Neutral Red dyes as a function of the applied potential. Error bars represent three experimental trials. The inset shows the experimental traces of both dyes at 2.5 kV. The arrow signifies the potential at which no signal increase was observed for Methyl Green while Neutral Red was being concentrated.

difference in threshold voltage of the dyes enables the selective exclusion of one small dye molecule while allowing another relatively similar molecule (in terms of molecular weight, charge, electrophoretic mobility, and diffusion properties) to pass. Therefore, at potentials below 3.0 kV, Neutral Red can be excluded (and concentrated) while Methyl Green passes from the reservoir.

The ability to exclude specific species simply by adjusting the potential has significant consequences. The development of a multi-gated (parallel or serial) device that can selectively exclude certain molecules and allows other to pass centers on the successful exclusion at an individual gate. By demonstrating separation of similar species at one gate, the device can be envisioned to include multiple gates either in series or in parallel to execute multiple separations simultaneously. Also, the establishment of distinctive threshold voltages demonstrates the dynamic control of the system with both the ability to target a particular species at each port or gate, as well as the capacity to collect either all or a small portion of a species of interest, simply by adjusting the electric field.

4 Concluding remarks

This work describes a new separations regime, termed electrophoretic exclusion, capable of differential transport of small molecules in bulk solution near the entrance to a capillary. The method counters hydrodynamic flow with the electrophoretic velocity of a species to concentrate an analyte near the entrance of a capillary. Proof of concept experiments using cationic Methyl Violet dye and neutral Martius Yellow dye show the ability to concentrate Methyl Violet while passing the neutral dye. A threshold voltage was demonstrated for exclusion that corresponds to the published models. By determining the threshold voltages of the cationic dyes Neutral Red and Methyl Green, the dyes were able to be differentiated using the electrophoretic exclusion technique. The capability to differentially separate species while remaining in bulk solution enables parallel and serial separation modes not available with other separations schemes.

The authors have declared no conflict of interest.

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