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Investigation of Electrophoretic Exclusion Method for the Concentration and Differentiation of Proteins

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This work presents a technique termed as "electrophoretic exclusion" that is capable of differentiation and concentration of proteins in bulk solution. In this method, a hydrodynamic flow is countered by the electrophoretic velocity to prevent a species from entering into a channel. The separation can be controlled by changing the flow rate or applied electric potential in order to exclude a certain species selectively while allowing others to pass through the capillary. The exclusion of various proteins is investigated using a flow-injection regime of the method. Concentration of myoglobin of up to 1200 times the background concentration in 60 s was demonstrated. Additionally, negatively charged myoglobin was separated from a solution containing negatively charged allophycocyanin. Cationic cytochrome *c* was also differentiated from a solution with allophycocyanin. The ability to differentially transport species in bulk solution enables parallel and serial separation modes not available with other separations schemes.

Proteins are typically studied in medical analysis because they divulge information about the physical state of an organ or biological system. However, they are often present in trace amounts or in complex fluids (such as serum, urine, or saliva). To overcome these challenges, separation and concentration techniques are employed. Some traditional methods utilized to isolate and concentrate proteins include ultrafiltration,[1] immunoassay,[2−4] and chromatographic processes such as size-exclusion[5,6] and affinity chromatography.[7−9] These methods have greatly contributed to protein analysis, but they have some limitations. For example, ultrafiltration is an off-line technique and can result in substantial sample loss, while immunoaffinity methods are time-consuming and expensive. The chromatographic strategies also have limitations, as affinity chromatography is expensive with the risk of column fouling, while size-exclusion chromatography has difficulty resolving species with similar molecular weights.

One area that has shown promise in the concentration and separation of proteins is electric field gradient techniques. Many of these methods, including electric field gradient focusing (EFGF), dynamic field gradient focusing (DFGF), and temperature gradient focusing (TGF), have been discussed in detail in various reviews.[10−15] Briefly, these techniques create a gradient in the electric field within a capillary, either by electrode placement or by using a temperature sensitive buffer and employing a counterflow to focus analytes.[16−21] Species are focused in the channel according to their differing electrophoretic mobilities. The Dovichi group originally proposed the potential of balancing electrophoresis with a counterflow to differentiate analytes with similar electrophoretic mobilities.[22] Significant experimental work in flow balanced capillary electrophoresis (CE) was then performed by the Jorgenson group.[23] These studies demonstrated how the efficiency and resolving power of CE could be improved by using the counterflow. To further elucidate the utility of flow counterbalanced CE, packed columns were used to resolve enantiomers and isotopes.[24] These critical studies have paved the way for current counterflow electric field gradient methods.

These gradient electrofocusing techniques have demonstrated success in both concentration and separation of proteins. The original work in EFGF gave modest enhancement by concentrat-

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ing proteins up to 3-fold in a few hours.[25] More recent work has shown concentration of enhanced green fluorescent protein (EGFP) 10 000 times in 40 min. Additionally, the method successfully separated EGFP, Oregon green lysozyme, and fluorescein isothiocyanate conjugate hemoglobin.[26] DFGF has also displayed similar success. Recent work has utilized a controllable dual-polarity electric field to differentiate negatively charged fluorescein isothiocyanate conjugate bovine serum albumin (FITC-BSA), R-phycoerythrin (R-PE), and allophycocyanin in one separation, positively charged Texas red conjugated ovalbumin and cationized ferritin in a different experimental trial, and oppositely charged R-PE and cytochrome *c*.[27] Lastly, with TGF, FITC-BSA was focused 200-fold in 2 min.[28]

These electrofocusing techniques have shown great promise for the concentration and separation of biochemical species. However, mostly all of them are performed within the constraints of the capillary or channel. Only two methods have demonstrated differential behavior at the entrance of the capillary, and these are multistep processes that also utilize separations or enhancement inside the capillary.[18,29] The method presented in this contribution, electrophoretic exclusion, is capable of differentiating and concentrating species in the bulk solution. The ability to differentiate analytes in the bulk solution enables the development of a device with multiple exclusion gates toward creating fast, simultaneous separations.

Electrophoretic exclusion has been described in detail in a previous contribution.[30] Briefly, hydrodynamic flow and electrophoretic velocity are opposed at the entrance of a capillary to create differential behavior in the bulk solution. Initial work by Polson et al. utilized this exclusion method and demonstrated the ability to exclude and concentrate 200 nm particles at the entrance of a 20 μm i.d. capillary.[31] In addition, Pacheco et al. modeled differential transport at the capillary entrance to investigate the various conditions that impact exclusion.[32] More recent work by Meighan et al. employed electrophoretic exclusion to concentrate and differentiate small dye molecules (MW 200−400).[30] In the present work, various proteins, including myoglobin, allophycocyanin, and cytochrome *c*, were investigated using the electrophoretic exclusion method. Myoglobin was concentrated almost 1200-fold in 1 min, while separation of the proteins was also demonstrated.

EXPERIMENTAL SECTION

Reagents. Tris (USB, Cleveland, OH), myoglobin (Sigma, St. Louis, MO), allophycocyanin (Anaspec, Fremont, CA), cytochrome *c* (Biovision, Mountain View, CA), hydrochloric acid, and sulfuric acid (both Mallinckrodt Baker, Inc., Phillipsburg, NJ) were used as received. Tris-HCl buffer was prepared to 1 mM concentration at a pH of 8.0 using 18 MΩ Milli-Q water and HCl. Protein solutions were prepared to 2.5 mg/mL in the 1 mM tris buffer unless otherwise noted.

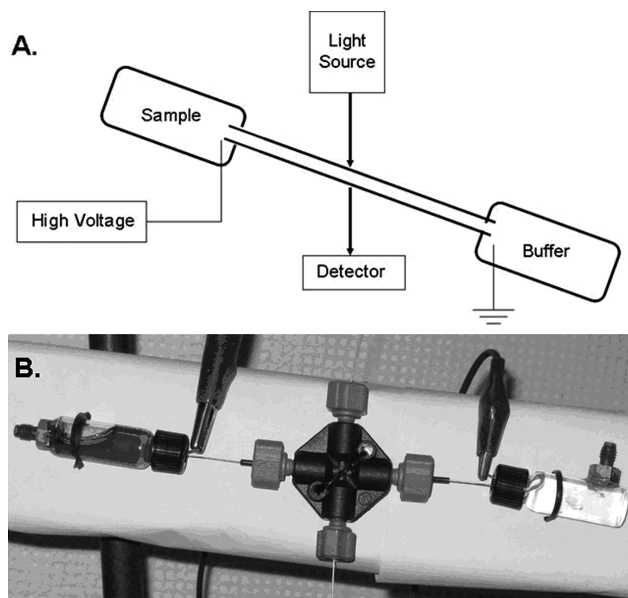

Figure 1. Instrumentation used for the exclusion method. Figure 1A is a schematic of the setup, while Figure 1B is a photographic image. A 10 cm capillary (75 μm i.d.) connects two modified reservoirs, and hydrodynamic flow travels from the sample vial (on the left) to the buffer vial (on the right). Detection is performed 5 cm from the entrance of the capillary using absorbance. Potential is applied at the entrance of the capillary, while the potential field in the reservoir remains flat.

Instrumentation. All experiments were performed using a electrophoretic exclusion apparatus described previously.[30] However, because of the change in analytes, some experimental modifications were made. Two modified 2 mL glass vials were connected using neutral-coated zero-EOF polyimide-coated fused silica capillaries (10 cm in length, 75 μm i.d., MicroSolv Technology Corporation, Eatontown, NJ). With the use of hot sulfuric acid, a window was burned midway on the capillary (~5 cm) to allow for absorbance detection. In addition, ~0.5 cm of the polyimide coating was removed at the end of the capillary so that an integral electrode could be formed by sputter-coating it with 30 nm titanium and then 50 nm platinum. The sputter-coated tip was 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 clear epoxy so that only the capillary tip was conducting and to ensure that a flat potential field existed in the reservoir.[31] This design allows the potential field to be initiated immediately at the capillary entrance.

The instrumental apparatus was mounted on a rotatable board so that the flow rate could be adjusted if necessary (Figure 1). The glass vials were modified so that they were open to air and solution 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 1.1 nL/s with a linear velocity of 0.026 cm/s. The flow was measured before and after experiments to ensure that it was maintained throughout the trials.

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Table 1. Comparison of Various Proteins, Their Electrophoretic Mobilities Using a Commercial CE and Electrophoretic Exclusion, and Their Threshold Voltages

protein	electrophoretic mobility CE ($cm^2/V\ s$)	electrophoretic mobility EE ($cm^2/V\ s$)	threshold voltage EE (kV)
cytochrome *c*	1.2×10^{-4}	5.6×10^{-5}	−1.5
myoglobin	-8.3×10^{-5}	-5.1×10^{-5}	2.5
allophycocyanin	-5.2×10^{-6}	-3.5×10^{-5}	5.0

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, a 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.

RESULTS AND DISCUSSION

Principles of Electrophoretic Exclusion. The electrophoretic exclusion method creates differential behavior at the entrance of the capillary by opposing fluid flow with electrophoretic velocity. In this contribution, a flow injection-like strategy is used to analyze the separation and concentration of various proteins. When the electrophoretic velocity is greater than or equal to the hydrodynamic flow velocity, exclusion occurs at the entrance of the channel.[30,32] Three critical parameters are required for exclusion: electrophoretic mobility of the analyte of interest, the hydrodynamic flow, and the applied electric field. The exclusion can be controlled by varying the flow and electric fields, as the electrophoretic mobility is constant for a set of experiments, defined by the buffer and the target species.

In order to differentiate two analytes under a fixed flow field, an electric potential must be chosen that is high enough to exclude a higher mobility species while allowing a lower mobility one to pass through the capillary. The most important parameter for generating this separation is the threshold voltage for each species. This experimental value is the potential at which exclusion is initiated at the entrance of the capillary and is obtained by applying increasing potentials until a signal response was observed in the trace. For the flow rate utilized in these experiments (1.1 nL/s or 0.026 cm/s), the proteins employed gave threshold voltages below a few kilovolts (Table 1). For each species, their electrophoretic mobilities were experimentally obtained on a commercial CE (Tris buffer at pH 8.0), and with the use of the measured flow rate and length of the capillary, the calculated mobilities using electrophoretic exclusion were determined (Table 1). The electrophoretic mobilities determined by CE were consistently larger than those calculated from exclusion data. In CE, the linear velocity remains constant, whereas in the exclusion method, the local linear velocity of the fluid is lower within the collapsing flow stream that is entering the capillary when compared to the velocity within the capillary. As the exclusion method gives lower mobilities than a commercial CE, it can be assumed that the lower mobilities are a result of the lower fluid velocities at the entrance of the capillary, and thus the bolus must be forming in the bulk solution. Aside from slight differences in local buffer conditions and possible surface interactions in the CE experiments, these results support the previous work in that the separation is occurring within the sample volume and not within the capillary.[30] Furthermore, the average radius of the collected bolus (and width of that bolus, using the raw data peak width) can be estimated by assuming a hemispherical collapsing flow geometry at the capillary entrance. With the use of the surface area of a hemisphere, the cross-sectional area of the inner diameter of the capillary and the volume flow rate, the radius of this bolus was estimated to be between $65 \pm 20\ \mu m$ with a volume of approximately 6×10^{-7} mL.

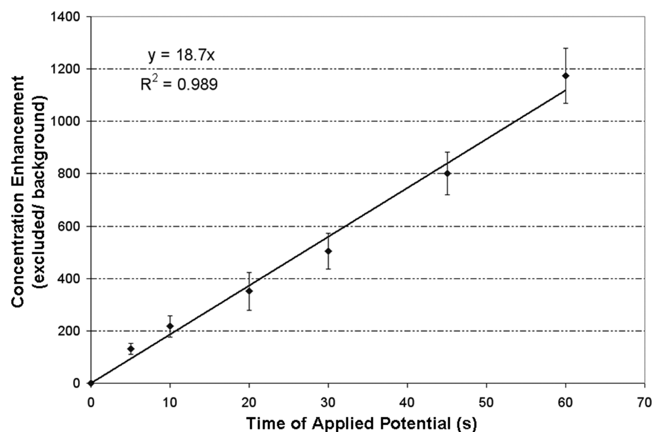

Figure 2. Average concentration enhancement of myoglobin at 3 kV. The time of applied potential was varied from 5 to 60 s, and the resulting increase in concentration is shown. The concentration enhancement was calculated by dividing the amount of material excluded with the method by the background. Each point represents the average of three trials.

Exclusion and Enhancement of Myoglobin. Previous work has demonstrated successful exclusion and differentiation of small dye molecules.[30] However, because of the relatively high diffusivity of the small molecules (on the order of 10^{-5} cm^2/s), only modest enhancement (∼40 times in 30 s) was achieved.[32] In this study, the ability to enhance the protein myoglobin (16.9 kDa) was examined. Myoglobin has a p*I* of 7.6, thus it has a negative charge at pH 8. Potential (3 kV) was varied between 5 and 60 s, and the resulting concentration enhancement is presented (Figure 2). Concentration enhancement is defined by dividing the amount of protein excluded (micrograms) by the background amount of protein (micrograms). These values were calculated by determining the concentration that corresponds to the obtained absorbance using the capillary diameter and molar absorptivity of myoglobin. With the concentration, flow rate of the system, peak width, and molecular weight of myoglobin, the amount of protein could be ascertained.

With the use of the exclusion method and an initial concentration of myoglobin of 2.5 mg/mL, enhancement of nearly 1200-fold was obtained in 60 s. In addition, a linear relationship exists between the length of time of applied potential and the enhancement. This linear relationship suggests that, within limits, the potential for the method to continue to concentrate species at the entrance of the capillary as long as potential is applied. With the use of this setup, the upper limit to the enhancement was not investigated, and as a consequence, possible negative effects, including protein aggregation, were not observed. Future device designs will incorporate a stirring mechanism to avoid analyte aggregation and prevent the alteration of localized fields at the

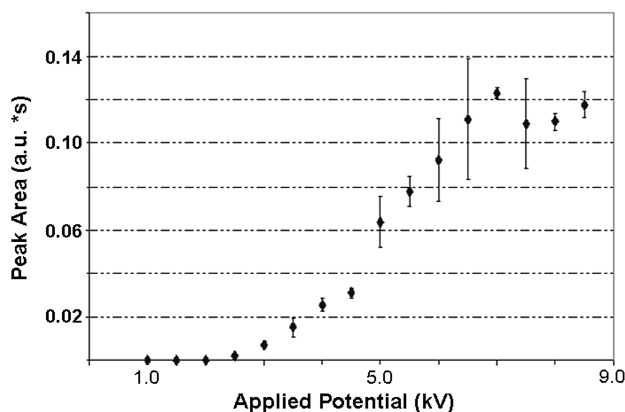

Figure 3. Exclusion profile of 2.5 mg/mL myoglobin. Potential was applied for 10 s from 1.0 to 8.5 kV increasing at 0.5 kV intervals. Error bars represent three trials.

capillary entrance as well as allow full examination of the capabilities of this approach. Compared to the other electric field gradient techniques, the enhancement generated with this method is competitive with several contributions reporting enhancements up to 1000-fold in 10−30 min for various proteins.[16,25,33−35] Furthermore, although the enhancement is similar, the time required is much lower than those reported for other gradient electrofocusing techniques. The method with the greatest enhancements (up to 10 000-fold) require long experimental trials (40 min to 4 h).[26,36]

What is also interesting to note is the difference in enhancement between small molecules and proteins. In 30 s, small molecules were concentrated nearly 40-fold,[30] while the proteins were concentrated almost 1200-fold in the same time. This discrepancy is considered to be the result of the differences in diffusivity between the species. The diffusion can be estimated by comparing the steady state Gaussian zones formed using $c/c_0 = \exp(ay^2/2D)$.[37] Assuming a diffusivity of 10^{-6} cm^2/s for small molecules and 10^{-8} cm^2/s for proteins, this estimation supports the difference in concentration between the species. Thus, although the method can prevent the small dye molecules from entering into the capillary, their high diffusivities result in only modest concentration enhancement. In addition, the ability of the technique to exclude both small molecules and larger proteins indicates the potential for the method to be successful with other species of interest, such as DNA or peptides.

The exclusion profile of myoglobin was investigated by applying 10 s of potential varied from 1.0 to 8.5 kV (Figure 3). No concentration is observed before 2.5 kV, the concentration steadily increases between 2.5 and 6.5 kV, and the amount of material concentrated is stable at potentials greater than 6.5 kV. At potentials below 2.5 kV, or the threshold voltage of myoglobin, the resulting velocity is insufficient to counter the flow into the capillary. The relatively slowly rising concentration enhancement is considered to be the result of the parabolic flow into the capillary and the opposing flat electrophoretic forces at the capillary entrance. At lower potentials, the molecules entrained in the slower edges along the walls of the capillary are being excluded, but the faster-moving species in the centerline of the parabolic flow are not. As the potential increases, more species can be excluded but only at higher potentials (>6.5 kV) can all of the myoglobin be excluded at the experimental flow rates.

Separation of Multiple Proteins. Once successful exclusion of myoglobin was demonstrated, the separation of two proteins was investigated. Again, the threshold voltage is the critical parameter for electrophoretic exclusion. The species with the lower electrophoretic mobilities will be trapped at higher threshold voltages. The proteins of interest for this study include myoglobin, cytochrome *c*, and allophycocyanin.

To demonstrate separation of two proteins, 1 mL of 2.5 mg/mL myoglobin (-8.3×10^{-5} cm^2/V s) and 1 mL of 2.5 mg/mL allophycocyanin (104 kDa, p*I* 4.2, -5.2×10^{-6} cm^2/V s) were combined, and 1.5 mL of the mixture was added to the upper reservoir. Myoglobin was monitored at 405 nm (black), while allophycocyanin was monitored at 650 nm (gray). Control experiments were performed to ensure that myoglobin gave no response at the 650 nm signal nor did allophycocyanin respond at 405 nm. Both of these proteins have a negative charge at the buffer pH, so normal polarity was utilized for the experiments. On the basis of the threshold voltages for both proteins (Table 1), 3.5 kV was chosen for differentiation. This potential is greater than the threshold value of myoglobin but less than that of allophycocyanin. Myoglobin was successfully excluded and collected at 3.5 kV for 10 s, as indicated by the peak, while allophycocyanin showed no response (Figure 4). To ensure that separation was occurring as a result of the difference in thresholds, and thus mobilities, different potentials were investigated (Figure 4, insets). When the potential was set to 2.0 kV, which is lower than both proteins' threshold voltages, no response was observed in either trace. Furthermore, when a potential was set that was greater than both thresholds (5.5 kV), a peak was observed in both traces, indicating that both proteins were excluded from the channel.

A signal decrease continues to be observed in all signal traces, which is visible in Figure 4. This signal decrease was discussed in a previous contribution,[30] and it is similar to migrating vacancy peaks in CE.[38−41] What is unique in this study, though, is that the signal decrease appears after the peaks, whereas in the previous contribution, it appeared before the peaks. Several experimental variables were altered, however, that could have contributed to the shift, including buffer, analyte, and capillary. Although we cannot yet fully describe this phenomenon, it continues to be consistent and controlled; therefore, a full characterization will be addressed in future research.

These experiments demonstrate the ability to manipulate the exclusion of a species simply by changing the potential applied at the capillary entrance. With the potential set higher than one threshold voltage (myoglobin) but lower than another (allophycocyanin), differentiation of the negatively charged proteins was

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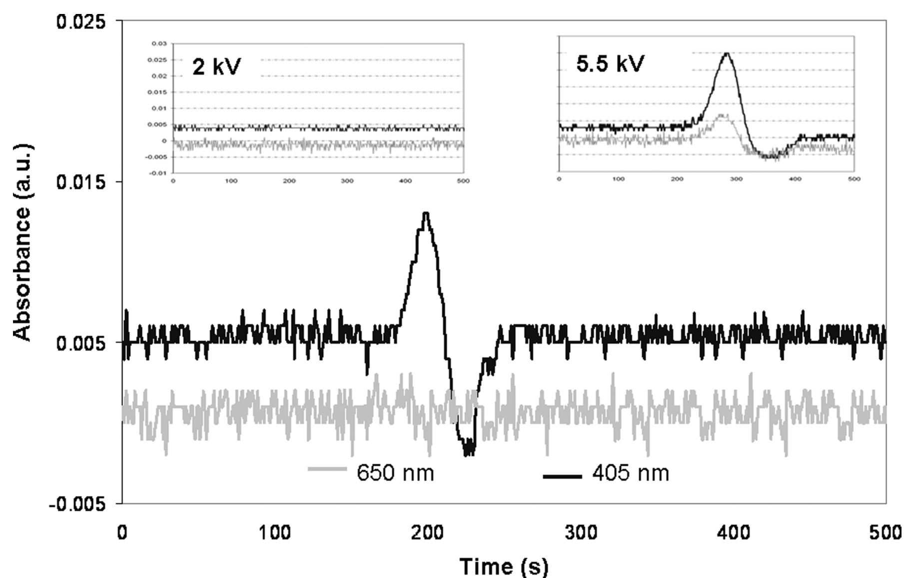

Figure 4. Separation of negatively charged myoglobin and allophycocyanin at 3.5 kV. Myoglobin was monitored at 405 nm (black), and allophycocyanin was monitored at 650 nm (gray). Potential was applied for 10 s. Insets: controls showing peak manipulation by altering the potential. The inset on the left shows no response when 2.0 kV was applied, while the inset on the right depicts peaks on each signal trace when 5.5 kV was applied.

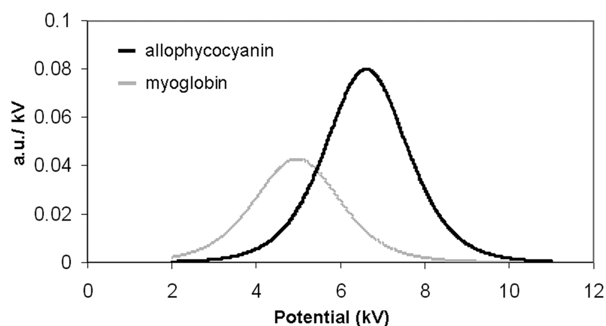

Figure 5. Peaks calculated as a result of differentiation with respect to potential. Myoglobin (gray) and allophycocyanin (black) are presented using the electrophoretic exclusion method.

attained. The controls shown in the insets further support the idea that certain species can be manipulated based on their mobilities. The low potential with no peak formation demonstrates the movement of both species into the next reservoir as the potential was unable to arrest either protein at the exclusion gate. In addition, both species were excluded by setting the potential higher than their respective threshold voltages.

Although this method does not provide a "traditional" resolution between species due to the nature of the separation in bulk solution, resolution can be examined. This technique is envisioned to be operated in parallel, with several capillaries set to exclude slightly differing species. These data can allow us to begin to envision how this highly parallel system might work. To determine resolution between analytes, exclusion profiles (such as Figure 3) were gathered for both proteins. The data were fitted using the least-squares curve fitting function in Matlab, and the best fit of the exclusion plots was found to be $f(x) = \{a/(b + e^{-cx})\}$. With these plots, data representing traditional electrophoretic peaks were generated by taking the derivative of the data with respect to potential (Figure 5). Both derivatives were plotted on the same graph, with allophycocyanin indicated by the black trace and myoglobin represented by the gray trace.

With the use of this representation, resolution between the peaks was calculated to be 0.64. Because this method does not provide a traditional electropherogram, in order to compare these results to a standard electrophoretic system, the peak widths were compared in terms of mobilities. With electrophoretic exclusion, the peak width is on the order of 10^{-5} cm^2/V s, while for comparable results in capillary electrophoresis of proteins, peak width can be as small as 10^{-7} cm^2/V s. Given that these experiments are performed with pressure-induced flow with the accompanying parabolic flow cross section (inducing Taylor dispersion), the results are reasonable. Although this resolution is modest, only two negatively charged species were investigated, and this resolution was for the experimental conditions presented. Noting the sources of "band broadening" here as Taylor dispersion, electric field inhomogeneities at the capillary entrance, and diffusion, there is an expectation that decreased capillary diameter and deterministic electrode placement will improve resolution dramatically.

Proteins with opposite charges were also investigated using the electrophoretic exclusion method. Allophycocyanin and cytochrome *c* (12.3 kDa, p*I* 10.7, 1.2×10^{-4} cm^2/V s) were examined. Cytochrome *c* was monitored at 405 nm (black), and allophycocyanin was monitored at 650 nm (gray). Again, a separation potential (−2.0 kV) was chosen based upon the threshold voltages of each protein, and potential was applied for 10 s. The 405 nm trace shows a peak, indicating exclusion of cytochrome *c*, while 650 nm shows no response (Figure 6). To demonstrate the lack of response below the threshold voltage, the potential was set to −1.0 kV, and neither trace showed a response (Figure 6, inset). The lack of a peak(s) was expected as −1.0 kV was below the exclusion threshold of cytochrome *c*.

The trials with allophycocyanin and cytochrome *c* demonstrate differentiation of two oppositely charged species at the exclusion gates. These results, combined with the differentiation of the negatively charged proteins, signify that the exclusion gates can be tailored to exclude positive or negative species simply by

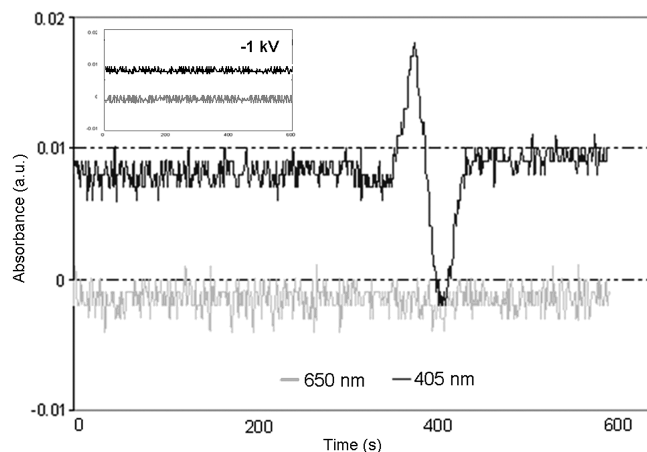

Figure 6. Separation of allophycocyanin and cytochrome *c* at −2.0 kV. Cytochrome *c* was monitored at 405 nm (black), and allophycocyanin was monitored at 650 nm (gray). Potential was applied for 10 s. A peak is seen at 405 nm at −2.0 kV, but no response is seen in either trace at −1.0 kV (inset).

reversing the polarity applied to the system. The ability to discriminate species in such a nature enables the development of a more intricate device with multiple exclusion gates in one sample reservoir.

Separation and concentration of both positively- and negatively charged species have been demonstrated by various groups.[27,42] Lin et al. utilized a tandem EFGF system for focusing,[42] whereas Burke et al. developed a DFGF device that can separate both types of analytes within the same channel.[27] However, employing one device for separation and concentration is advantageous because of the capability to have dynamic control over the separations. In addition, the ability to differentially separate species in the bulk solution enables parallel and serial separation modes not available with other separations schemes. As an example with myoglobin and allophycocyanin, the potential can be set to 3.0 kV and concentrate purely myoglobin using electrophoretic exclusion. With the development of a device with multiple exclusion gates with each gate tuned to a different potential, several species can be concentrated simultaneously.

To further demonstrate the unique capability of the electrophoretic exclusion method, manipulation of two proteins was investigated (Figure 7). With the use of the negatively charged allophycocyanin and myoglobin, the potential was held at 5.5 kV. As shown in Figure 4, this potential was high enough to block both proteins from entering. The potential was held at 5.5 kV for 20 s and then lowered to 3.0 kV for 20 s before the potential was removed. Once the potential was reduced to 3.0 kV, the trapped allophycocyanin was released, as indicated by the peak that appears first in the gray trace. After the potential was removed, the excluded myoglobin enters the capillary. These results demonstrate the dynamic control that can be achieved using the electrophoretic exclusion method, as the potential can be altered midtrial to collect as much or as little of a species as desired.

(42) Lin, S.-L.; Li, Y.; Tolley, H. D.; Humble, P. H.; Lee, M. L. *J. Chromatogr., A* **2006**, *1125*, 254–262.

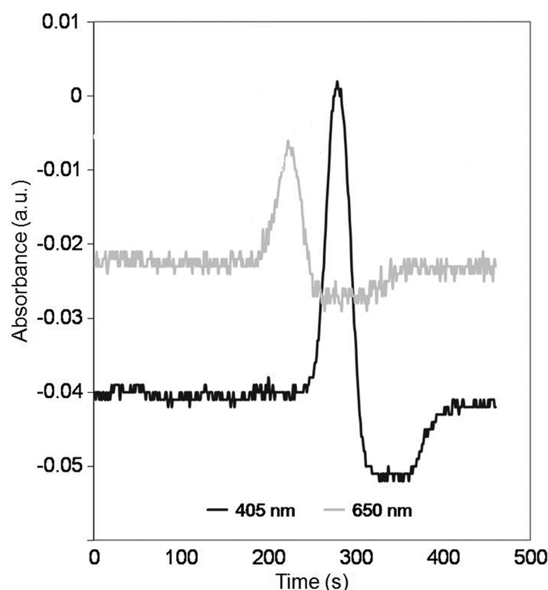

Figure 7. Dynamic and species-specific bolus formation and release. The potential was set to 5.5 kV for 20 s, which blocked both proteins, then lowered to 3.0 kV for 20 s to release allophycocyanin before the potential was removed. Myoglobin was monitored at 405 nm (black) while allophycocyanin was monitored at 650 nm (gray). The allophycocyanin peak is observed before the myoglobin peak.

CONCLUSIONS

This paper describes the concentration and differentiation of proteins using electrophoretic exclusion. With the use of the method, myoglobin was able to be concentrated 1200 times in 1 min. The key parameter for exclusion, and thus differentiation, is the threshold voltage of a species, which is dependent upon the analyte's electrophoretic mobility. In this work, the threshold voltages of three proteins (allophycocyanin, myoglobin, and cytochrome *c*) were ascertained. With the use of these obtained values, the potentials applied could be tailored to create differential behavior. To demonstrate this behavior, negatively charged myoglobin was excluded from the capillary while allophycocyanin was not. The accumulation of these species could also be manipulated midcollection. In addition, two oppositely charged proteins (allophycocyanin and cytochrome *c*) were separated using the method. The capability to differentially separate species while remaining in bulk solution enables parallel and serial separation modes not available with other separations schemes.

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