Digitally Controlled Electrophoretic Focusing

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Proteins can be simultaneously separated and concentrated by applying a constant force and opposing this with a gradient in a second force. In this work, a constant hydrodynamic force is opposed by a gradient in the electric field which allows charged molecules to focus in order of their apparent electrophoretic mobilities. The electric field gradient is established and maintained using an array of electrodes whose voltages are individually monitored and adjusted by a computer-controlled circuit board. The computer-generated electric field gradient allows charged molecules to be focused without using a pH gradient. Since the proteins are not focused at their pI's, precipitates do not form, so focused concentrations in excess of 50 mg/mL are not unusual. In addition, since the field shape is dynamically controlled from the computer on a point-by-point basis, the field profile can be adjusted during a run to improve the resolution. In this paper, the column and controller are described together with experimental results and a model which illustrates the separating power and flexibility of this technique.

More than a decade ago, O'Farrell described how proteins could be focused at the interface between two different gel filtration media packed into the upper and lower halves of an electrochromatography column. His results were soon replicated by others who found that at least one protein, ferritin, could be concentrated beyond 100 mg/mL. This remarkable feat was tempered by the finding that his approach worked poorly with protein mixtures and would be difficult to scale up. Nevertheless, O'Farrell had found a way to focus proteins in an electric field that did not require the use of a pH gradient. This suggested that other approaches to electrophoretic focusing might be found.

Recently, Koegler and Ivory demonstrated that charged proteins could be separated and focused using an electric field gradient in an electrochromatography column. A fluided cooling jacket was used to form a linear gradient in the electric field which drove the proteins against a constant flow of buffer in a packed dialysis tube. This approach was slow and cumbersome and gave mediocre results, but it successfully illustrated an alternative focusing technique which we named electric field gradient focusing (EFGF).

Next, Greenlee and Ivory showed that proteins would focus in the electric field gradient formed by an axial conductivity gradient and opposed by a constant flow of buffer. Greenlee's apparatus was far simpler to build and operate than was Koegler's. It was also surprisingly fast when run in free solution, reaching equilibrium in less than 10 min, and gave unexpectedly good results when filled with a 40-μm size exclusion (SEC) packing. The success of Koegler's and then Greenlee's approach to EFG encouraged us to attempt focusing using an array of electrodes to establish the electric field gradient.

EXPERIMENTAL SECTION

Apparatus. The focusing chamber (Figure 1A) was formed from two blocks of 15 × 6 × 1.2 cm³ Plexiglas and a 0.3-cm-thick Teflon spacer. The front block, which houses the separating column, has a 8 × 0.1 × 0.05 cm³ trough machined into it. The rear block, which houses the 50 controllable electrodes, has a 4.8 × 0.3 × 1.5 cm³ trough and the spacer has a 6.5 × 0.2 cm² slot machined through it. The trough in the front block is isolated from the spacer by a dialysis membrane and is packed with a 4.5-μm NovaPak Diol chromatography media (Waters). The rear trough and slot admit a recirculating buffer that may have the same composition as the running buffer, acts both as coolant and anolyte, and removes electrolysis products from the electrode array. Since the coolant is in contact with the column via a dialysis membrane, it can also be used to dialyze the running buffer to exchange salts or other low-molecular-weight solutes. The coolant inlet and outlet lines are visible at the top of Figure 1A.

Outside of the focusing chamber, the coolant buffer is circulated through a glass heat-exchange reservoir submerged in an ice bath. From here the coolant is introduced into the bottom of the focusing chamber and is passed over the electrodes at ~15 mL/s using a centrifugal pump (Cole-Parmer). A syringe pump controls the flow of the running buffer through the packed bed at 15–150 μL/h. The running buffer enters the column in the upper flow inlet on the front face and exits from the lower flow outlet on the front face (Figure 1A). All lines are PEEK with flangeless fittings; sample is loaded through a 10μL loop on a six-port injection valve (Upchurch).

Controller. The 50 chamber electrodes are made from 0.25-mm-o.d. platinum wire (Aldrich Chemical), are mounted in the rear Plexiglas block with a 0.05-in. pitch, and are connected to a SCSI ribbon cable using SM5-series microstrips (Samtec). Each of the SCSI leads is connected to its own printed-circuit (PC) monitor/controller board (Figure 1B) mounted on the wire wrap...
motherboard. Each monitor/controller board is segregated into three areas: high voltage, monitoring, and control. The high-voltage area isolates the chamber electrode voltages, which can be as high as 600 V, from the relatively sensitive electronics used to measure and adjust the electrode voltages. The monitor area of each PC board scales down the electrode voltage by $\frac{1}{100}$ and sends this signal to a commercial thermocouple board which digitizes the signal before sending it to the computer. The computer scans all 50 electrodes, compares these readings with the programmed profile, and sends a digital signal to a set of 50 DACs which tell the optical isolators to adjust the effective resistance of high-voltage line to reduce the departure of the measured electrode voltages from the programmed voltage profile. A complete scan/control cycle of the 50 controllers is taken every second. Each of the 50 controllers is mounted vertically on a wire-wrapped motherboard; power to the controllers' motherboard is drawn from the computer. A 600-V power supply (Xantrex) provides current to the column's 50 high-voltage electrodes via the 50 voltage controllers.

**Procedure.** After the recirculating coolant has reached operating temperature and the packed column has been cleaned, e.g., with 7 M urea, and equilibrated with running buffer, 10 µL of protein solution is injected into the column, which has a packed volume of 28 µL exposed to the 50 controlled electrodes, using a standard sample loop. Before protein reaches the outlet, the controller is booted using a default voltage pattern and the power supply is brought up to a voltage in the range 200–600 V. The operator then selects the initial electric field gradient, and the computer program adjusts the electrode voltages until this gradient is attained, typically less than 5 min from a “cold” start.

**RESULTS**

Typical results with the chamber are presented in Figures 2–4, which are digitized images of naturally colored or artificially labeled proteins dynamically focused in an electric field gradient. In each of these figures, the flow in the packed section of the column is from top to bottom and the voltage gradient is greatest near the outlet, vanishes at the inlet, and is linear over the 2.5-in. length of the electrode section. In all of the experiments reported here, the first 49 array electrodes are anodes while the last electrode is a cathode set to ground and the electric field strength is linear.

The proteins (all purchased from Sigma) and run conditions used in these experiments are listed in Table 1. Individual protein bands (Figure 2a–c) take 10–30 min to focus depending on the flow rate of the running buffer. The bands formed have roughly the baseline width predicted by the linear theory discussed in the next section of this paper and reach concentrations in the range of 5–50 mg/mL even without subtracting the nonaccessible volume of the packing. For example, in Figure 2a, the phycoerythrin band is less than 0.2 mm thick, 1.0 mm wide, and 0.5 mm deep and contains 2.5 µg of protein which translates to an apparent focused concentration of roughly 25 mg/mL.

When multiple proteins are run, as is the case in Figure 2d, it is sometimes difficult to set a linear field gradient where all of the proteins can be retained in the column and baseline separated.
at the same time. This is due in part to the wide variation in mobilities in this particular group of proteins and, to a greater extent, to the tendency of the concentrated protein bands to merge into isotachophoretic bands if they come too close to one another.

**THEORY**

Most, if not all, members of the family of electrophoretic focusing techniques can be described by the simple flux equation,

\[ N_{p,x} = -D_p \frac{dc_p}{dx} + \left( \langle u_{p,x} \rangle + z_p \omega_p \right)c_p = 0 \]  

where \( N_{p,x} \) is the molar flux of protein along the \( x \)-axis of the electric field. For focused protein bands, the flux is set equal to zero to indicate that the bands are stationary. Equation 1 is composed of a dispersive term, a convective term, and an electrophoretic term where \( c_p \) is the protein concentration, \( D_p \) is a diffusion or dispersion coefficient, \( \langle u_{p,x} \rangle \) is the apparent hydrodynamic velocity along the \( x \)-axis, \( z_p \) is the protein charge, \( \omega_p \) is the protein mobility, \( I_x \) is the current density, and \( \sigma \) is the electrical conductivity. For proteins to focus, it is necessary that at least one of the terms in parentheses varies so that their sum forms a gradient which vanishes at a discrete point in the chamber and which pushes the protein toward that point regardless of its initial location. Focusing occurs at the point in the chamber where the sum of the terms in parentheses vanishes.

Setting the sum of the terms in parentheses in eq 1 equal to zero, it is seen that focusing may be accomplished in at least five different ways: (a) in a pH gradient with \( u_p = 0 \), proteins focus at
the point where the net charge on the protein vanishes, i.e., $z_p = 0$, as is the case with isoelectric focusing (IEF); (b) in a gradient in $u_{p,1}$ with $z_p$, I, and $\sigma$ held constant, which corresponds to O’Farrell’s counteracting chromatographic electrophoresis; (c) in a gradient in $\omega_p$ with $u_{p,2}$, $z_p$, I, and $\sigma$ constant, e.g., focusing a protein in a urea gradient. With $u_{p,2}$ held constant, proteins can be focused by (d) forming gradients in $I$, as was done by Koegler and Ivory; or (e) forming gradients in $\sigma$, as was done by Greenlee and Ivory. Both of these approaches generate gradients in the electric field and the ions in solution, it cannot accurately describe overlapping or contiguous bands. A more detailed version of this model that can handle these situations is given by Koegler and Ivory.6 Both of these approaches generate gradients in the electric field similar in many respects to the gradients generated by the instrument described in this paper.

Setting $I_x = I_{x,1} + I_{x,2}$ to form a linear gradient in the current, the focal point is found at

$$x_f = -\frac{(u_{p,2})^2}{Z_p\omega_p(1)} + \frac{I_{x,1}}{I_{x,2}}$$

and, integrating eq 1, the concentration is given by

$$c_p = \frac{M_f}{W} \sqrt{\frac{Z_p\omega_p(1)}{2\pi\sigma D_p}} \exp\left[-\frac{Z_p\omega_p(1)}{2\sigma D_p}(x - x_f)^2\right]$$

which yields a Gaussian distribution in the focused band. The standard deviation, $\chi$, of the peak around the focal point is then

$$\chi = \sqrt{\sigma D_p/Z_p\omega_p(1)}$$

where $M_f$ is the total mass in the focusing chamber and $W$ is the perimeter of the chamber. Note that focused bands are made thinner by low conductivities and steep current gradients. Conversely, resolution, $R$

$$R = \frac{1}{2} \sqrt{\frac{z_{p,2}^2}{Z_p\omega_p(1)} - \frac{1}{\sqrt{Z_p\omega_p(1)}} - \frac{1}{\sqrt{Z_p\omega_p(1)}}}$$

is improved by reducing the gradient, raising the conductivity, and increasing the velocity of the running buffer. The simple linear model presented above does a good job of predicting protein location and baseline width when bands are completely resolved. However, since it ignores nonlinear coupling between the electric field and the ions in solution, it cannot accurately describe overlapping or contiguous bands. A more detailed version of this model that can handle these situations is given by Koegler and Ivory.7

**Simulation.** The linear model can be used to explore the advantages of electronically controlled focusing, specifically, by adjusting the field parameters to enhance resolution during a run. Consider the case, for instance, in which five recombinant protein isoforms with the electrophoretic mobilities given in Table 2 are focused near the top of the DFGF chamber (Figure 5). These proteins might first be moved as a unit to the center of the chamber, e.g., by increasing the flow rate, and then spread over the entire length of the column by expanding the electric field so that the fastest peak is near the chamber inlet and the slowest peak is near the outlet (Figure 6). By flattening and reducing the electric field gradient, the three low-mobility peaks could be eluted from the chamber while the two fastest peaks are retained. After switching to step changes in the electric field the remaining two

<table>
<thead>
<tr>
<th>Figure</th>
<th>Proteins (Sigma)</th>
<th>Catalog no.</th>
<th>pH</th>
<th>VE (V/cm²)</th>
<th>Flow (μL/h)</th>
<th>Applied voltage (V)</th>
<th>Protein mass loaded (μg)</th>
<th>Load protein conc (mg/mL)</th>
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<tr>
<td>2a</td>
<td>(R)-phycocyanin (PC)</td>
<td>P 0159</td>
<td>7.0</td>
<td>13.0</td>
<td>44</td>
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<td>(R)-phycocyanin (PC)</td>
<td>P 1536</td>
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<td>13.0</td>
<td>42</td>
<td>300</td>
<td>5.0</td>
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<td>Carbonic anhydrase (CA)</td>
<td>C 6653</td>
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<td>39</td>
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<td>M 9267</td>
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<tr>
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<td>Bovine serum albumin (BSA)</td>
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<td>(R)-phycocyanin (PC)</td>
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<td>3.7</td>
<td>138</td>
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*Conditions: 10 mM tris-phosphate buffer on 4.5-μm NovaPak-Diol packing.*
peaks, whose mobilities differ by ~3% can be completely separated and individually eluted from the chamber (Figure 7).

CONCLUSIONS
These results demonstrate that it is possible to establish and manipulate an electric field gradient by using a computer-controlled electrode array. In combination with a continuous flow of buffer, this gradient can be used to simultaneously separate and focus proteins as well as other charged molecules at concentrations in excess of 50 mg/mL in a packed-column format.

It should be noted that DFGF cannot replace IEF as an analytical technique: it cannot work at the isoelectric point (pI) because the proteins’ mobilities vanish at that point. However, it does effectively extend the pH range over which focusing can take place to include native buffers as well as non-native, denaturing, and reducing conditions. A resultant advantage is that focusing can be accomplished away from a protein’s pI, thus avoiding the precipitates that often form near the isoelectric point and making it preferable to IEF as a preparative technique.

Though all of the work presented here was done using linear electric field gradients, the software can be revised to allow point-by-point adjustment of the field including reversing the field to aid in elution of fractionated bands, isolating and mobilizing a single protein band, or stepping the gradient to improve processing capacity. In addition, since the electronic controller and the DFGF technique is largely independent of chamber capacity, there is no reason it cannot be applied to other types of electrophoresis equipment operating at larger or smaller scales.

Finally, this work was done with colored and labeled proteins to avoid the cost of installing on-line detectors during early trials with the column. However, there is no reason optical or other detectors could not be mounted on the chamber to provide real-time monitoring of the separation. This would open up the possibility of having the computer detect the various peaks, optimize the separation by locally adjusting the field gradient to tease refractory proteins apart, and then pull off those peaks that were selected by the operator either before or during a separation. This is planned for future work.

ACKNOWLEDGMENT
This research was supported in part by NSF Grant BES-9417239. Materials, supplies, and suggestions were graciously provided by Immunex Corp., Seattle, WA, without whose support this work could not have been completed. A proprietary Nova Diol packing was graciously donated for this work by Waters Corp. Important contributions to the development of the electronic controller were made by Fred Scheutze of WSU Technical Services, Alan Guyer of WSU EECS Computer Maintenance Shop, and J. R. Yates of WSU’s EECS Electronics Shop.

Received for review October 22, 1998. Accepted January 30, 1999.
AC981160K