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Dynamic Isoelectric Focusing for Proteomics

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Dynamic isoelectric focusing is a new technique that is related to capillary isoelectric focusing but uses additional high-voltage power supplies to provide control over the shape of the electric field within the capillary. Manipulation of the electric field changes the pH gradient, enabling both the location and width of the focused protein bands to be controlled. The proteins can be migrated to a designated sampling point while remaining focused, where they can be collected for further analysis. This ability to collect and isolate the protein bands while maintaining a high peak capacity demonstrates that dynamic isoelectric focusing has great potential as a first dimension in a multidimensional separation system. Dynamic isoelectric focusing can achieve a peak capacity of over 1000, as shown by both mass spectrometry analysis and direct imaging.

The complex nature of the proteome requires separation methods with high resolution and dynamic range to enable comprehensive analyses. Proteomics has traditionally been done using 2-DE,[1] which separates using isoelectric focusing in the first dimension followed by electrophoresis.[1,2] It is estimated[3] that 2-DE has a peak capacity of ∼10 000. 2-DE has several limitations, including difficulty in analyzing very small, large, acidic, basic, or hydrophobic proteins, and it has poor reproducibility.

There is an increased interest in developing methods to replace 2-DE to enable a more comprehensive analysis of proteomic samples, but individual separation methods lack the necessary peak capacity. Liquid chromatography and capillary electrophoresis typically provide a peak capacity of less than 200, so they are often combined with other separation techniques to increase the total system peak capacity. Some examples are LC/CE[4−6] and LC/LC.[7−12] The combination of two orthogonal separation methods greatly increases the total peak capacity of the system, but the interface between the two techniques is often complex.[13,14] The gel format of 2-DE overcomes this problem by allowing the entire sample to be separated by the second dimension simultaneously. Liquid-based techniques, however, are usually forced to have the second dimension sequentially analyze fractions as they elute from the first dimension. This increases analysis time and often forces the second dimension to trade resolution for speed to complete the separation before a new fraction is ready.

The challenge exists to develop a method to be used as a first dimension that does not suffer from these drawbacks. Ideally, it would provide a high peak capacity with a high sensitivity, the solvents and flow rate would be compatible with potential second dimensions, and it would be easily interfaced to other analysis methods. One method that meets many of these requirements is capillary isoelectric focusing (cIEF). It allows for the separation of intact proteins in their natural form and provides a peak capacity >1400.[15] Because it is a focusing method, the separated proteins are stationary, providing as much time as required between fraction collections. This allows much more flexibility in the speed of the second dimension.

The major drawback to cIEF is the difficulty in interfacing it to other separation methods without sacrificing resolution. The protein bands are very narrow when they are focused and are difficult to extract without significant broadening. Often, the proteins are forced out of the capillary through the use of pressure,[16,17] which greatly distorts the bands and leads to a very low peak capacity. There are other methods for the mobilization of focused protein bands that can provide a higher peak capacity, such as salt mobilization[18] and electroosmotic zone displacement,[19] but each has several drawbacks. Since these methods migrate the focused proteins out of the capillary using electroosmotic flow, the collection of very narrow bands and the speed at which the bands must be collected are major challenges. The peak capacity obtained after mobilization is still much lower than the peak capacity measured with static focusing systems.

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Other focusing methods have been implemented and evaluated by various groups. Recently, Lee and co-workers have demonstrated a method that uses an electric field gradient and an opposing hydrodynamic flow to separate charged analytes. This method has been named electric field gradient focusing.[20,21] The analytes migrate until they reach a point where their electrophoretic velocity matches that of the bulk liquid flow. This method has been able to concentrate proteins ~10 000 times.[22] A major drawback of this system is that it is difficult to find the optimal operating conditions for the electric field against the hydrodynamic flow due to the field's lack of homogeneity. The system has also encountered problems with dispersion, leading to issues with resolution and sensitivity.[23] The fabrication of the separation channel has also proved more challenging than desired.

Another approach to increasing the sensitivity and resolution of protein separation was demonstrated by Ross and Locascio.[24] They developed a system to balance the electrophoretic velocity against the bulk flow of the solution in the presence of a temperature gradient, known as temperature gradient focusing. This method requires that the temperature dependence of the buffer conductivity differ from the conductivity of the analyte. Therefore, for a given analyte, the buffers that can be used are extremely limited, and the separation is limited by the temperature constraints of both the analytes and the buffers. This limits the range of mobilities that can be separated. The advantage to this method is that it has shown to be capable of a 10 000-fold concentration increase of a given analyte, but it still lacks the versatility and capacity needed for proteomic investigations.

Ivory and co-workers have done much work in the area of instrumentation for proteomic research, including the development of a digitally controlled electrophoretic focusing system.[25] The addition of multiple electrodes in the system allowed for the protein bands to be sharpened and moved. The design of the system limits its usefulness, because the number of electrodes present limits its resolving power, and the fabrication of the system is labor intensive.

Dynamic IEF is similar to cIEF in that it focuses proteins on the basis of their isoelectric point by establishing a pH gradient using ampholytes and an electric field. Although the pH gradient and the electric field in cIEF are constant, giving each focused protein in the sample a fixed position, dynamic IEF moves the pH gradient through manipulation of the electric field. This enables control over each protein's position and focused width. By changing the electric fields during a separation, focused proteins can be brought to a fixed sample point for collection and further analysis by chromatography or mass spectrometry, for instance. The dynamic control of each protein's position overcomes the major disadvantage of cIEF, which is the difficulty in interfacing to other techniques, while providing all of the advantages. This paper explains the general theory behind dynamic isoelectric focusing, shows the experimental setup, and then presents data supporting the theory and illustrating the capabilities of this method that make it useful as part of a multidimensional separation system.

THEORY

Dynamic IEF establishes a pH gradient in a capillary using carrier ampholytes and an electric field, as does standard cIEF. Whereas cIEF uses a single high-voltage power supply at one end of the capillary, dynamic IEF incorporates additional power supplies along the capillary length. These power supplies permit the shape of the electric field within the capillary to be changed. The effects that a different electric field shape has on the pH gradient within the capillary can be understood using fundamental IEF equations introduced by Svensson.[26]

The base differential equation that determines the concentration distribution of an ampholyte is

$$\frac{Cui}{q\chi} = D\frac{\mathrm{d}C}{\mathrm{d}x} \tag{1}$$

where C is the ampholyte concentration, u is the electrophoretic mobility in $cm^2\ volt^{-1}\ s^{-1}$, i is the current in amperes, q is the cross-sectional area of the capillary in cm^2, χ is the conductance of the buffer in $\Omega^{-1}\ cm^{-1}$, D is the diffusion coefficient in $cm^2\ s^{-1}$, and x is the distance along the separation space. Each side describes the mass flow per second and cm^2, with the left side being mass flow due to migration in the electric field and the right side, due to diffusion. In the same reference, a solution to this differential equation gives the concentration profile of a focused analyte as

$$C(x) = C_0 \exp\left(-\frac{pix^2}{2q\chi D}\right) \tag{2}$$

where p is the change in electroosmotic mobility with distance ($\mathrm{d}u/\mathrm{d}x$). This equation indicates that the peak has a Gaussian shape, with spread about the isoelectric point given by

$$\sigma = \sqrt{\frac{q\chi D}{pi}} \tag{3}$$

Since an increase in the applied voltage increases both i and p, the width of each ampholyte band changes linearly with respect to electric field strength.

During dynamic IEF, the electric fields encountered by the ampholytes are changed during the analysis, with the goal of controlling the shape of the pH gradient. When the electric fields change, the ampholyte peaks will correspondingly change their width, as eq 3 indicates. This change of bandwidth could happen in one of two ways. One option could be that the amount of overlap between adjacent ampholytes will simply change, and the pH gradient will remain stationary. Ampholytes in a region where the electric field decreased would overlap the adjacent ampholytes more, for instance. If this is the case, then the pH gradient is stationary, and the electric field strength will have little effect on the analysis.

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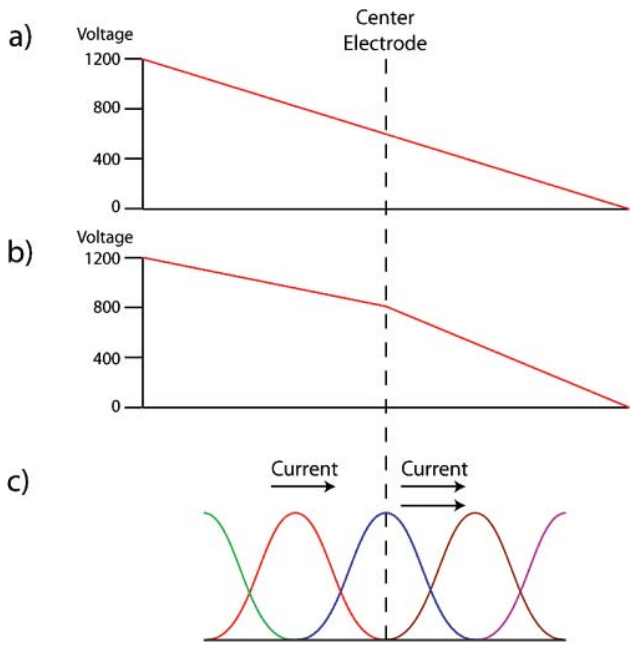

Figure 1. Example of a three-electrode dynamic IEF setup with (a) equal currents through the whole capillary, (b) unequal currents, and (c) the effect of unequal currents on an ampholyte peak.

The other possibility is that the ampholytes maintain the same amount of overlap with each other, even under different field conditions. For this to happen, the entire pH gradient would need to shift with the electric field as each ampholyte band either contracts or expands. Dynamic IEF is based on the idea that this is what happens when the electric fields are changed. We can determine that this is what will occur by considering the behavior of an ampholyte that crosses the middle electrode during a change in electric field strength.

Suppose that we set up a dynamic IEF experiment as illustrated in Figure 1a using voltages of 1200 (voltage 1), 600 (voltage 2), and 0 V (ground), with voltage 2 being in the middle of the capillary. The electric field strength will be constant across the whole capillary. Since the voltage drop between voltages 1 and 2 is the same amount as between voltage 2 and ground, the current in both parts of the capillary is the same. This means that voltage 2 does not actually supply any current to the capillary. The ampholyte bandwidths will also be the same along the capillary length.

If we raise the value of voltage 2 to 800 V, then we force a piecewise linear electric field to form in the capillary, as shown in Figure 1b. There is a voltage drop of 400 V on the high-voltage side and 800 V from the middle to ground. The current from voltage 1 to voltage 2 is, therefore, much less than the voltage 2 to ground. The extra current flowing to ground comes from voltage 2 and has a large effect on the ampholytes.

Imagine a particular ampholyte band that crosses the center electrode. When voltage 2 is increased, part of the band will have a current flow through it higher than the rest, as illustrated in Figure 1c. The additional current will cause an increase in the charge state of those molecules, making them migrate toward ground. This phenomenon is easier to understand when we consider that the main charge carriers in IEF (after the ampholytes are focused) are protons. As the ampholytes begin to migrate away from the electrode, they will leave a region of reduced ampholyte concentration near the electrode. The molecules on the other side of the electrode will move across, fill the gap, and begin their own migration due to the higher current and proton flux. In this way, ampholyte peaks can cross the electrode and move closer to the ground electrode. Though this example has a peak centered around the electrode, the same effects and results apply to any peak that crosses the electrode.

Under the increased field, every ampholyte will narrow and provide the space for the additional ampholytes. The migration and narrowing of the ampholytes causes a corresponding shift in the pH gradient and an increase in the concentration of ampholytes in the region of higher electric field. This process will continue, creating an increasing difference in the ampholyte concentration on each side of the electrode. A steady state will eventually be reached when the number of molecules migrating across the electrode is equal to the number diffusing in the opposite direction, as indicated in eq 1. This result clearly shows that migration will cause the pH gradient to shift in response to the electric field.

One verification of this prediction can be acquired by simply monitoring the currents during a dynamic IEF analysis. Although the ampholytes in a standard IEF separation are migrating to their isoelectric point, they carry charge, and the observed current is high. As they focus, the current drops to a low background level. This is often the easiest way to determine that the separation is complete. In a dynamic IEF experiment, we should observe a similar behavior in the current as ampholyte repositioning occurs.

In the example from Figure 1, the initial focusing current from voltage 1 would be high and then drop off to a background level when focusing was complete. Voltage 2 would provide very little current during this time. When voltage 2 is increased, it would cause many ampholytes to migrate toward ground as the pH gradient shifts, so a high current from voltage 2, followed by a drop off to a background level should be observed. At the same time, the current from voltage 1 should decrease but show no large changes. The currents would only behave like this if the pH gradient is shifting with the electric field. This behavior of the power supply currents during focusing and refocusing is observed exactly as described and is discussed in the results section, confirming the migration of the pH gradient with a change in the electric field. Although this theory is currently not complete, it does illustrate the driving force behind the pH gradient shift and should suffice while further research is being performed in this area.

The theory indicates that dynamic isoelectric focusing will provide the same peak capacity as cIEF, which is ~1400. The advantage that dynamic IEF has is that this separation power can be effectively interfaced to a second dimension because each focused band's position and width can be controlled through the electric field. For efficient coupling to a second dimension, the peak width should be the same size as the collection window. A typical cIEF band is 100 μm wide in the capillary and has a volume of <1 nL, making it very difficult to isolate a single peak. Dynamic IEF can create wide peaks without sacrificing resolution by decreasing the electric field strength at the sampling point, making extraction and isolation much simpler and more efficient.

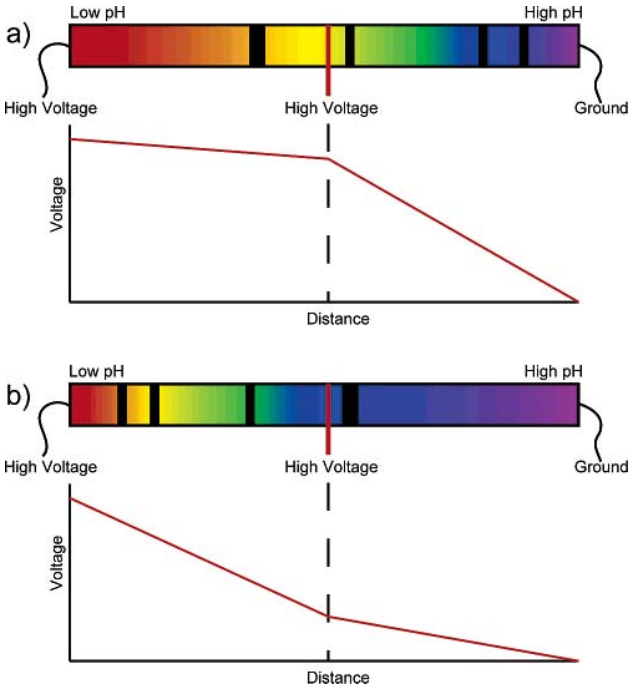

Figure 2. The electric fields and pH gradient in a three-electrode system with (a) a high voltage and (b) a low voltage on the middle electrode.

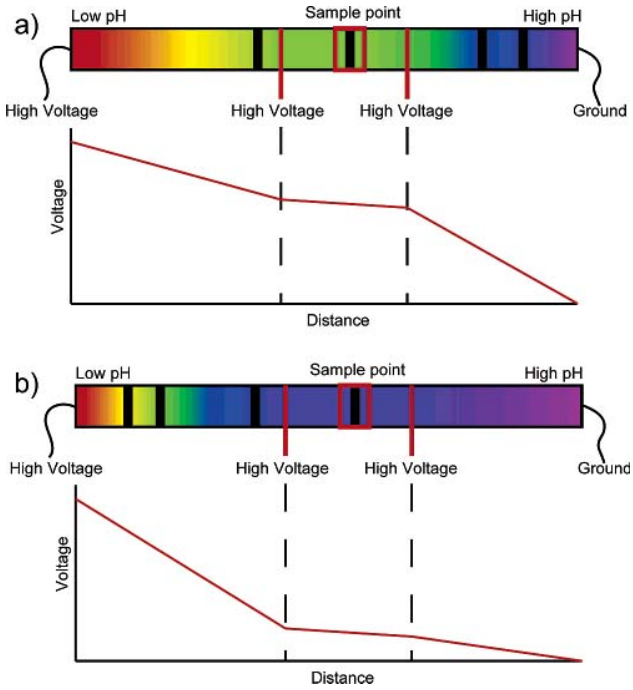

Figure 3. The electric fields and pH gradient in a four-electrode system with (a) high voltages and (b) low voltages on the middle electrodes. Note that the voltage difference and, therefore, the bandwidth between the middle electrodes are constant.

A simple implementation of dynamic IEF is provided in Figure 2. It uses only one additional power supply, so it is referred to as a three-electrode system. This simple system allows for dynamic mobilization to be observed and tested. Though the theory and implementation of dynamic IEF can be validated using this setup, it does not allow for easy sample collection or interfacing to additional separation methods. A fixed sampling point could be integrated, but it would not provide consistent collection efficiency. Imagine that a sample collection valve is placed in the system to the right of the middle electrode in Figure 2a. The band collected under conditions depicted in Figure 2a will be very narrow due to the steep electric field and pH gradient. However, when the conditions in 2b are reached, then the band at the valve will be much broader. If all other conditions remain constant, then the width of each band is based on the electric field strength, as was shown in eq 3. Since the change in voltage and pH with distance is much lower in Figure 2b than in 2a, the band will be broader. This variance in field strength and the corresponding change in bandwidth decrease the usefulness of a three-electrode system. A four-electrode system can solve this problem, as illustrated in Figure 3.

In a four-electrode system, there are two additional electrodes in the capillary that have a fixed observation or sampling point between them. Their voltages are adjustable, but the voltage difference between them remains constant. A fixed voltage differential provides a constant electric field strength at the sampling point, meaning that the bands focused between these electrodes will also have a constant and controllable width. The total voltage at the sampling point will change, changing the pH, but it is the electric field strength that determines the width of the band. Figure 3a shows the collection of a protein with a low pI, and 3b shows the collection of a high pI. The bandwidths at each end of the capillary will change with changing conditions, but the center section will always encounter constant widths.

MATERIALS AND METHODS

Reagents and Chemicals. Acetic acid, ammonium acetate, hydroxypropyl cellulose (HPC, av MW 100 000), HPLC grade dimethyl sulfoxide (DMSO), HPLC grade water, PTFE tubing, horse heart myoglobin, α-cyano-4-hydroxycinnamic acid (CHCA), universal indicator solution, and phosphate-buffered saline (PBS, pH 7.4) were obtained from Fisher Scientific (Fairlawn, NJ). Ampholytes (Pharmolyte 3-10 for IEF) and calibrated protein mixture (broad range, pH 3−10) were obtained from Amersham Biosciences (Piscataway, NJ). Tetramethylrhodamine-5-(and-6)-isothiocyanate (5(6)-TRITC) was obtained from Molecular Probes (Eugene, OR).

Sample Preparation. Fluorescently labeled protein solution was prepared according to the manufacturer's protocol. Briefly, a standard mixture of proteins with known isoelectric points was dissolved in PBS at 6 mg/mL. TRITC was dissolved in DMSO at 1 mg/mL. The TRITC solution was added to the protein sample and incubated in the dark for 2 h. The tagged protein solution was then filtered through a 100-kDa centrifugal filter (Millipore Bedford, MA) to remove any protein aggregates. Finally, the unbound TRITC was removed from the tagged proteins by filtering through a 10-kDa centrifugal filter (Millipore) and using the retained portion.

Samples for analysis by MALDI-MS were mixed with 1 mg/mL CHCA matrix solution in a sample/matrix ratio of 1:8. After mixing, 0.5 μL of this solution was spotted onto a MALDI target plate and allowed to dry.

Instrumentation. The high-voltage power supply used was fabricated in-house. It contains five 10-kV supplies (Ultravolt,

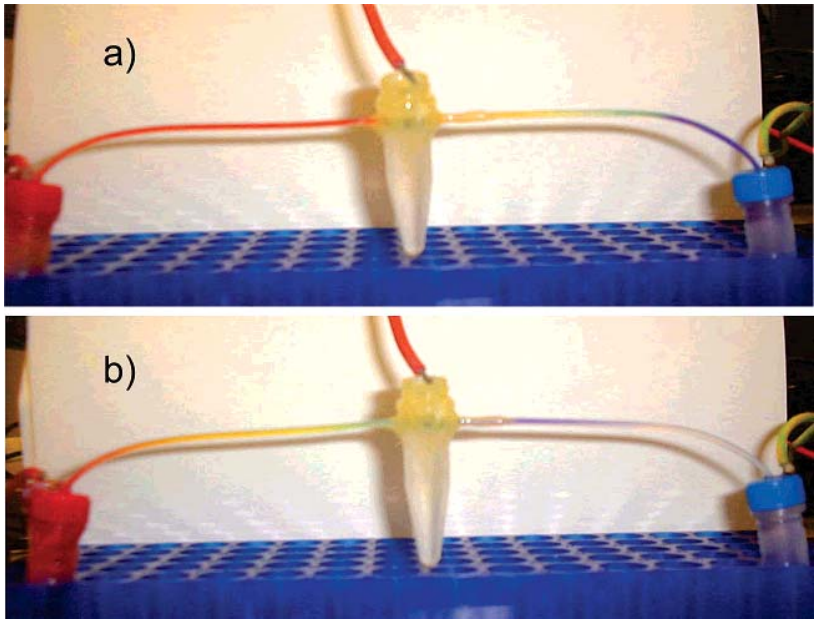

Figure 4. Photographs of dynamic isoelectric focusing in a polymer tube with universal indicator added to the ampholyte mixture. (a) Initial focusing with voltages of 2200, 2195, and 0. (b) Refocusing with voltages of 2200, 1100, and 0. The repositioning of the pH gradient is clearly seen.

Ronkonkoma, NY), and each is individually controlled by a computer using LabView (National Instruments, Austin, TX).

Fluorescence emission was viewed using an inverted microscope with a 10× long-range objective with excitation at 544 nm and emission at 572 nm. A digital video camera was attached to the microscope for recording images and video.

The MALDI-MS used was a 4700 TOF-TOF (Applied Biosystems, Framingham, MA) with a pulsed nitrogen laser set at an intensity of 4000. It was operated in linear mode with a focus mass of 17 000 Da.

Focusing Capillaries. Fused-silica capillaries of 75 μm i.d., 360 μm o.d. (Polymicro, Phoenix, AZ) were coated with hydroxypropyl cellulose by filling them with a 5% HPC solution and then baking them at 200 °C for 45 min.[27] This effectively eliminates electroosmotic flow in the capillary. Each capillary was filled with either 1% ampholyte solution or a protein sample solution in 1% ampholytes prior to use.

Electrical Connections. To perform dynamic IEF, electrical connections must be made to different locations inside the capillary. This must be done without causing leakage of the ampholyte solution and without forming bubbles in the capillary. There are several ways of doing this,[28,29] but for this research, a simple connection was sufficient. A PTFE tube with an inner diameter of 0.014 in. (~356 μm) was carefully cut partway through orthogonal to its length. The capillary was then cut, and the two sections were inserted into the tubing until their junction was 1 mm from the cut. The cut section of the tubing was then inserted into a small acrylic reservoir that contained a platinum electrode connected to the high-voltage power supply. The lack of pressure coupled with the offset between the cut and the capillary union permitted an electrical connection while preventing fluid leakage.

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Safety. These experiments involve high voltages that can be dangerous if proper precautions are not observed. Great care should be taken to avoid touching high-voltage electrodes and to prevent them from coming in contact with anything that is grounded.

RESULTS AND DISCUSSION

Proof-of-Concept with PTFE Tubing. Since dynamic IEF is a new implementation of an existing method, we took several different approaches to evaluate it. Proof-of-concept tests were performed using 0.02-in. (~528 μm) PTFE tubing in a three-electrode configuration. The tubes were filled with universal indicator and a 3% ampholyte solution. Photographs of this setup are seen in Figure 4. The relatively large tubing has several disadvantages, such as excess heating, siphoning, and eddy currents, but the size made it possible to easily see the pH gradients as they formed and were then moved. The anode contained 0.2 M acetic acid; the cathode, 0.5% ammonium hydroxide; and the center vial, 3% ampholyte. Figure 4a shows the initial focusing conditions with voltages of 2200, 2195, and 0 V. As expected, the pH gradient forms between the center electrode and ground, and the high voltage section is completely acidic. The center voltage was then dropped to 1100 V, and the gradient was moved so that a neutral pH was present in the center. This can be seen in Figure 4b. The refocusing took ~10 min. Although this experiment does not measure the performance of dynamic IEF for protein separations, it does verify the concept of dynamically controlling the pH gradient through the electric fields.

Monitoring Current Profiles. Another means of monitoring the focusing and refocusing process is through the currents from the power supplies. In isoelectric focusing, the total amount of current in the system drops exponentially as the ampholytes and proteins migrate to their isoelectric point. Dynamic isoelectric focusing should exhibit such a current drop every time the voltage is changed as the ampholytes migrate to their new positions. This

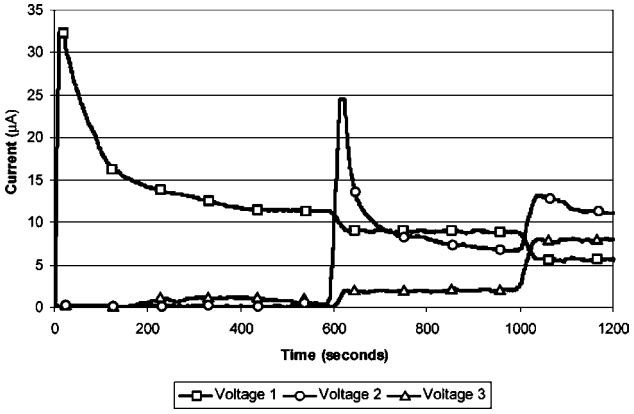

Figure 5. The current trace from a four-electrode system undergoing focusing with mobilization steps occurring at 600 and 1000 s.

expected behavior can be observed in the voltage and current traces of a four-electrode system with a total capillary length of 18 cm, consisting of sections of 7, 4, and 7 cm, respectively. The anode contained 0.2 M acetic acid; the cathode, 0.5% ammonium hydroxide; and each of the center vials had 3% ampholyte. Initial focusing was performed, followed by several mobilization steps. The recorded current trace is shown in Figure 5.

The starting voltages were 2400, 200, and 180 V for voltages 1−3. The final reservoir was always grounded. The initial focusing occurred mainly between voltages 1 and 2 because of the large voltage differential and took approximately 9 min. The proteins were considered focused when the current from voltage 1 reached its background level of ~4 μA/kV. Once the initial focusing was complete, voltages 2 and 3 were increased to 1200 and 1180 V to refocus the proteins to a different location inside the capillary. The current from voltage 2 rose and then decayed, indicating the refocusing that occurs. Focusing was performed a third time by increasing voltages 2 and 3 to 2100 and 2080 V. Total analysis time was ~20 min.

Testing pH in Sampling Region. Since there could be other processes responsible for the behavior of the currents, the pH in the center section of capillary was directly measured under similar conditions. Voltage 1 was set to 2000 V with a buffer solution of pH 3. The ground electrode had a buffer with a pH of 10, giving a pH span of 7 units. Initial focusing was performed by setting voltage 1 to 2000 V and the rest of the voltages to ground. This caused the entire pH gradient to form between voltages 1 and 2. After focusing, voltages 2 and 3 were increased, and the system was allowed to refocus. The voltages were then disconnected, and the center section of capillary (~4 cm in length) was removed. The liquid in this section was added to 50 μL of universal pH indicator solution. The center capillary was refilled with ampholyte solution and reinserted. The voltages were reconnected, and voltages 2 and 3 were increased, changing the pH within the center section. This whole process was repeated nine times, as listed in Table 1, with center voltages ranging from 15 to 89% of voltage 1, corresponding to pH values from 3.8 to 9. The expected pH at each center electrode was calculated by dividing the electrode voltage by the total voltage and then multiplying by the pH span of the separation. This result indicates the number of pH units between the electrode and ground. The final step is to subtract this value from the pH at the ground electrode. The color produced after each mobilization step matched the expected indicator color, confirming that the pH present at the sampling point is directly controlled by the electric field and can be changed as desired.

Table 1[a,b]

voltage 2	pH 2	voltage 3	pH 3	av pH	color	
					expected	observed
300	8.95	280	9.02	8.985	blue	blue
480	8.32	460	8.39	8.355	blue-green	blue-green
660	7.69	640	7.76	7.725	green-blue	green-blue
920	6.78	900	6.85	6.815	green	green
1100	6.15	1080	6.22	6.185	yellow	yellow
1320	5.38	1300	5.45	5.415	orange-yellow	orange-yellow
1480	4.82	1460	4.89	4.855	orange	orange
1620	4.33	1600	4.4	4.365	orange-red	orange-red
1780	3.77	1760	3.84	3.805	red	red

[a] Voltage 1 = 2000; pH 1 = 3; pH ground = 10. [b] pH = pH ground − pH range × voltage/(total voltage).

Monitoring Mobilization of a Focused Protein Band. A more direct validation of the theory was achieved by analyzing a protein mixture and directly viewing the mobilization of a focused protein band. This was performed using an inverted microscope and a fluorescently labeled mixture of proteins that had a wide range of pI values. Figure 6 shows one of the proteins, horse heart myoglobin, before, during, and after mobilization. From left to right, the pictures show the focused, immobile protein, the protein moving out of the view window, the protein slowing down as it reaches a new position, and the protein refocused ~1.5 cm from where it started. Note that the viewing window was moved between the second and third frames and that the width of the protein band is the same before and after mobilization. A video in the online supporting material shows this mobilization. Although it is difficult to get an accurate measurement of the bandwidth, the 75-μm internal diameter of the capillary gives an estimate of 125 μm. The protein experienced a constant electric field of 40 V/cm, so with a total system voltage of 1 kV, the estimated peak capacity is over 1000.

Multidimensional Separation Using Dynamic IEF. To demonstrate dynamic IEF in a simple multidimensional separation, the analytes in the center section of the capillary were collected and analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). This basic setup remained a four-electrode system as described, with the primary difference being that the capillary section between voltages 1 and 2 was initially filled with a sample of 1 mg/mL horse heart myoglobin in phosphate-buffered saline solution with 1% ampholyte. After focusing was finished, the liquid in the center section was removed, mixed with CHCA matrix and spotted onto a MALDI target. This permits us to identify the analyte present in the center section under various focusing voltages. By monitoring the changes in focused proteins under different conditions, we can obtain an estimation of the minimum peak capacity. For example, if the voltages are changed by 1% of the total system voltage and there is a different protein focused within the center section, then the peak capacity is at least 100. The actual peak capacity is still not known, but a lower limit can be found.

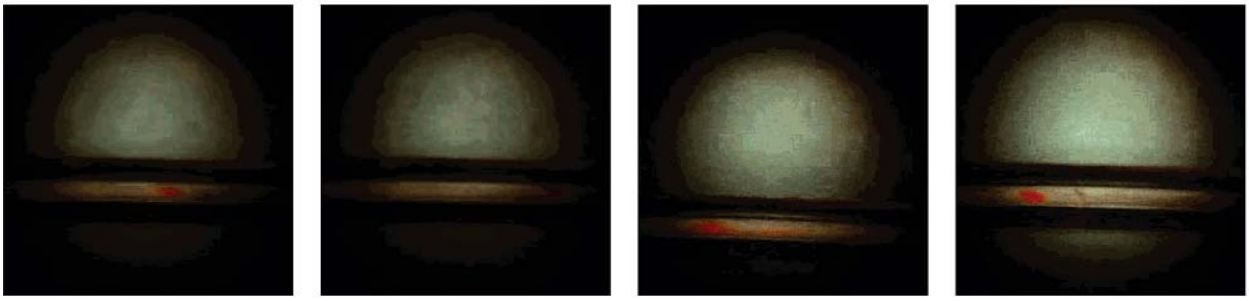

Figure 6. Pictures of a focused protein band during mobilization at time = 0, 15, 250, and 300 s. The microscope viewing area has been moved ~1.5 cm to the right for the final two images so that the refocused protein would be within the viewing window. The image color has been adjusted for increased visibility of the protein.

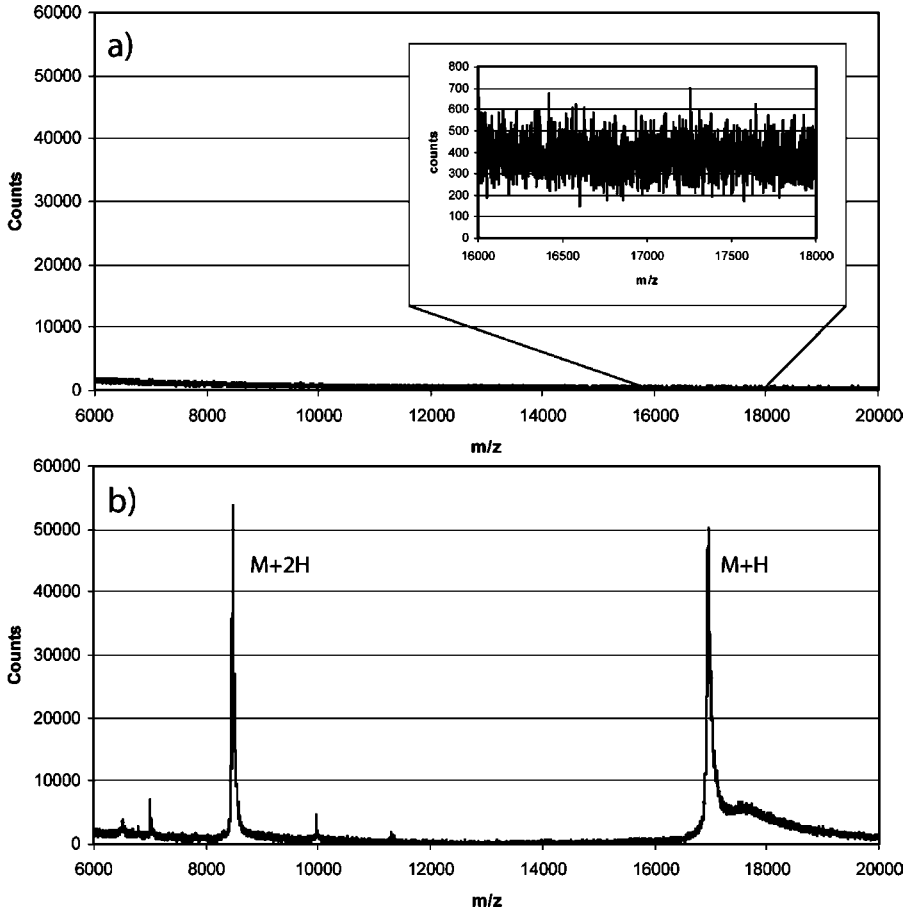

Figure 7. MALDI mass spectra of the contents of the center capillary in a four-electrode system. (a) voltages of 2200, 1002, 992, 0; and (b) 2200, 1004, 994, 0. The myoglobin peak at 16 964 *m*/*z* is undetectable under the first conditions and has over 50 000 counts under the second.

Figure 7a shows the mass spectrum with voltages of 2200, 1002, and 992 V. The myoglobin peak is absent. When both of the center voltages are increased by 2 V to 1004 and 994 V, the spectrum in 7b is obtained, showing a strong myoglobin peak. This experiment was repeated four times to demonstrate reproducibility. The replicates with the lower voltage setting did not have a detectable myoglobin peak either individually or with the spectra combined. The higher voltage setting produced myoglobin peaks with average intensities of 40 000 counts and a S/N of >250. This result indicates that at least part of the myoglobin peak is present within the center section after a voltage change of <0.1% of the total voltage. When the center voltages are increased a little more, the myoglobin peak does not increase in size (data not shown), indicating that the entire myoglobin peak was completely focused within the center section after the first increase in voltage. This shows that dynamic IEF can provide a peak capacity of at least 1100 and can maintain this when interfaced to a second analysis method. Although the 4-cm length of the capillary section that is removed limits the attainable resolution of this setup, it does illustrate the great potential that dynamic IEF has.

CONCLUDING REMARKS

Dynamic IEF is a new technique that generates a dynamic pH gradient in a capillary for the separation of proteins that provides many advantages over current methods. It is rapid and simple and can provide a high peak capacity that can be interfaced with other analysis methods. In addition to proteomic applications, dynamic IEF has valuable uses as a fractionation device. Its peak capacity permits the collection of very narrow (<0.01 pH units) bands of proteins that are difficult to obtain using other methods. This would permit the screening of a complex mixture for proteins that cause a certain type of biological activity, such as inducing cellular growth or death. Dynamic IEF could first be used to fractionate the sample to be tested for activity. When the desired activity is observed, then the specific fraction could be further fractionated and analyzed to identify the proteins responsible. This would permit rapid and comprehensive screening for certain types of protein activity and would simplify identification of those proteins.

Future work includes the integration of an automatic sampling valve between the two middle electrodes. This can be a standard HPLC injection valve that has internal volumes that are in the correct range. It is necessary that it also have a liquid flow path that is entirely a nonconductive polymer to prevent interference with the applied voltages. The integrated sample valve will be used to more easily interface dynamic IEF to other analysis methods, such as liquid chromatography or capillary electrophoresis. The high peak capacity of dynamic IEF coupled with its fast separation speed will allow a multidimensional system that has capabilities beyond 2-DGE and other current systems. Additional study of the theory of dynamic IEF and its relation to the established IEF equations is needed and will also be pursued.

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SUPPORTING INFORMATION AVAILABLE

A Quicktime video is available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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