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A Brief Review of Alternative Electrofocusing Techniques

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ABSTRACT

Isoelectric focusing (IEF) is an excellent tool at analytical scales but has some drawbacks at preparative and process scales. Alternative electrofocusing methods have been around for over a decade but have only recently reached the point where they can begin to compete head-to-head with IEF. This paper describes some of the advances made in this field since the mid-1980s and shows how they are related to IEF by a common mathematical expression. In addition, one new technique is described which allows real-time computer-control of the focusing gradient.

BACKGROUND

The first attempts in the mid-1950s at electrofocusing proteins in a pH gradient have been credited to Kolin (1). To do this, he established a pH gradient using solutions which were acidic at the anode of his device and alkaline at the cathode. When a protein solution was placed in this gradient and a current passed through the solution, each protein focused at that point in the chamber where the pH of the solution rendered it a net charge of zero, i.e., at its isoelectric point (pI). The arrangement of the pH gradient between the electrodes was such that a protein starting from any point in the chamber would migrate to its pI and form tight bands with concentrations typically in excess of 1 mg/mL. The Achilles' heel of this technique was that pH gradients formed from simple buffers were difficult to stabilize over the period of time required for focusing.

Modern isoelectric focusing (IEF) began with the synthesis of pH gradient-stabilizing ampholines by Vesterberg (1) and, with the arrival of agarose and

polyacrylamide gels, it soon became a standard analytical technique for protein characterization (2–4). On these IEF gels, commercially available narrow-range ampholytes can resolve proteins whose pIs differ by less than 0.05 pH unit. The newer immobiline gels can resolve proteins down to 0.001 pH units (5–7).

Attempts to adapt ampholyte-based IEF to industrial scales have met with limited commercial success: Fawcett (8) was the first to use a thin-film, free-flow electrophoresis device (9, 10) to perform continuous IEF. However, under the conditions he used, IEF takes several hours to reach its pseudoequilibrium state. The requisite low flow rates needed for single-pass focusing make this device susceptible to unstable natural convection and, therefore, difficult to scale beyond the milligram per hour range.

In 1979 Bier (11, 12) introduced Recycling Isoelectric Focusing (RIEF) which used a chamber 1–2 cm thick divided into 10–20 compartments by nylon mesh screens with 10–25 μm openings. At the start of a focusing run the chamber and connective tubing are filled with 1–2% ampholyte solution plus protein, a multichannel peristaltic recycling pump is turned on, and an electric field is applied across the chambers. The multichannel pump circulates the contents of each compartment through individual heat exchange tubes, spectrophotometric cells, and other peripheral equipment until it is eventually returned, i.e., recycled, back to its individual compartment through an inlet at the *top* of the focusing chamber (13).

RIEF introduced a number of innovations, the most important being that recycle coupled with external cooling would allow the use of relatively high electrical power densities and, as a result, shorter run times than Fawcett's device. In addition, the device capacity could be easily scaled by increasing the volumetric holdup of the recycle lines,* internal cooling coils could be incorporated into a large-scale electrophoresis chamber, additional compartments could be added to the processing column to improve resolution, and the chambers could be scaled by at least a factor of 10 by increasing the cross-sectional area of the screens. At least one paper (13) reported the RIEF as processing a gram of protein in an hour.

Since the introduction of the RIEF, several other preparative IEF devices have entered the market including BioRad's Rotofor (14), Rainin's RF3, and MinipHor (15). While each of these instruments has various strengths and weaknesses that are inherent in their design, they all use IEF which imposes limitations of its own, particularly at preparative and large scales. These are: 1) many solutes have low solubilities at their isoelectric points; 2) entire classes of solutes cannot be focused by this method either because they de-

* Doubling the volume doubles the run time, but this allows larger volumes to be processed in a single run.

grade at their isoelectric point (pI), e.g., nucleic acids, or they do not have a readily accessible pI, e.g., polystyrene latexes; and 3) isoelectric focusing is inherently a batch technique which, like chromatography, is difficult to make continuous at large scale. In addition to these shortcomings, if ampholytes are used to stabilize a pH gradient, they contribute up to $500 per gram of protein purified to the cost of focusing *and* they must be completely removed from parenteral therapeutics.

The problems encountered with soluble ampholytes has encouraged development of an instrument which uses immobilized pH gradients, i.e., "Immobilines" in Hoefer's IsoPrime (16), IEF focusing buffers using "defined" focusing media, i.e., the "ampholyte-free" Optifocus and Rotolyte buffer systems, and at least one free-flow apparatus which uses a thermal gradient that shifts the p*K* of a simple buffer to produce a stable pH gradient (17). While these and other developments in IEF have increased its breadth of application, the field of electrofocusing has recently expanded to include other methods besides IEF.

AN ALTERNATIVE TO IEF

The first instrument which allowed electrofocusing of proteins at pHs removed from the isoelectric point was developed by O'Farrell (18) and was named counteracting chromatographic electrophoresis (CACE). In his apparatus solute could be focused at the interface between two different gel filtration media packed into the upper and lower halves of an electrochromatography column (Fig. 1A). The upper half of the column was filled with a low-molecular weight size-exclusion (SEC) resin, e.g., BioRad P-10, and the lower half with a high molecular weight SEC resin, e.g., BioRad A-50m; a flow of running buffer was directed down through the column and the electric current oriented so that electrophoretic migration was against this flow. The flow was then adjusted so that a charged molecule would migrate toward the packing interface from either end of the column. The focused protein forms a dense band of concentrated protein with a sharp upper edge and a diffuse mass-transfer tail at its lower edge (Fig. 1B).

O'Farrell's results were soon replicated in other labs (19, 20) where it was found that at least one of the half-dozen or so proteins O'Farrell had worked with, specifically ferritin, could be brought to an apparent concentration beyond 100 mg/mL. This remarkable feat was tempered by the finding (21) that this approach worked poorly with protein *mixtures* and would be difficult to scale up due to its inherently low electrophoretic process speeds and Joule heating of the chromatographic bed. 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 raised the possibility that other approaches to electrophoretic focusing without a pH gradient might be found.

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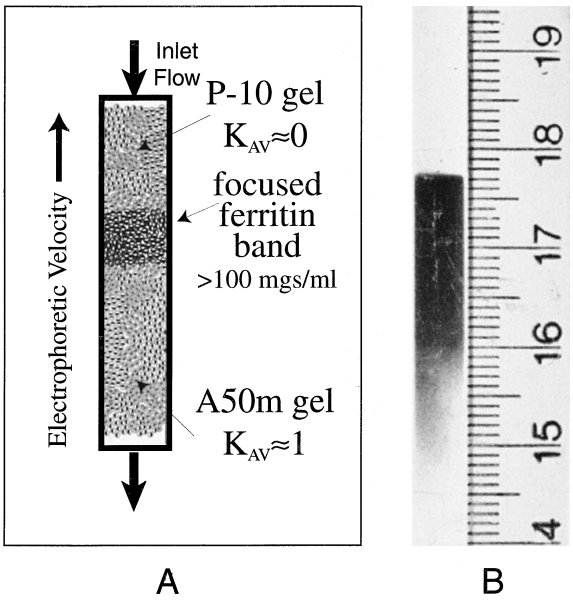

FIG. 1 (A) Illustration of the CACE setup used by O'Farrell. A low molecular weight size exclusion gel is poured on top of a high molecular weight size exclusion gel and solute, in this case ferritin, which focuses near the interface between the gels. (B) 10 mg equine ferritin (Sigma) focused in a CACE apparatus. The run conditions are: 1200 V (0.890 mA) applied over a 50-cm column, 10 mM Tris-acetate at pH 7.4, 0.01 mL/min in a 6-mm ID liquid-cooled glass tube. The gel interface is at 16 cm on the ruler, and the nominal concentration of the focused band is about 20 mg/mL in this experiment. This experiment was performed in my laboratory by Bill Gobie.

UNIFYING THEORY

The search for new members of the family of electrofocusing techniques begins with the flux equation

$$N_x = -D\frac{dc}{dx} + \left(u_x + z\omega\frac{I_x}{\sigma}\right)c = 0 \qquad (1)$$

where N_x, the molar flux of solute along the x-axis of the electric field, is set equal to zero in Eq. (1) to indicate that the solute is not moving. Equation (1) is composed of a dispersive term, a convective term, and an electrophoretic term where c is the solute concentration, D is a dispersion coefficient, u_x is the apparent chromatographic solute velocity along the x-axis, z is the solute charge, ω is the solute's absolute mobility, I_x is the current density, and σ is the buffer's electrical conductivity. In order for solutes to focus it is necessary that at least one of the terms in parentheses varies so that the sum of the terms in parentheses is a line with a value of zero at a single point along the x-axis. This will become the focal point for that solute. Furthermore, the gradient must be oriented so that it pushes each solute toward its focal point regardless of that solute's initial location in the column.

Setting the sum of the terms in parentheses in Eq. (1) equal to zero, one sees that electrofocusing may be accomplished in at least five different ways:

Type 1: In a pH gradient with $u_x = 0$, solutes will focus at the point where the net charge on the protein vanishes, i.e., $z = 0$, as is the case with IEF
Type 2: In a gradient in u_x with z, I, and σ held constant, which corresponds to O'Farrell's (18) CACE
Type 3: In a gradient in I, with u_x, z, and σ constant
Type 4: In a gradient in σ, with u_x, z, and I held constant
Type 5: In a gradient in ω with u_x, z, I, and σ constant, e.g., focusing a protein in a concentrated urea gradient

This simplistic view of focusing, which does not account for nonlinear ionic coupling, may overlook other members of the electrofocusing family but it does provide a starting point for identifying alternative electrofocusing techniques. If the term in parentheses is replaced with a generic linear gradient, $-mx + b$, the focal point, x_f is given by the formula

$$x_f = b/m, \qquad \text{where } m > 0 \tag{2}$$

The concentration profile, which turns out to be Gaussian in this linear example, is

$$c = \frac{M_T}{wt}\sqrt{\frac{m}{2\pi D}}\exp\left[-\frac{m}{2\pi D}(x - x_f)^2\right] \tag{3}$$

where M_T is the total mass of a solute loaded into the chamber, w is the chamber width, and t is the chamber thickness. The spatial standard deviation, χ, of the peak around its focal point is

$$\chi = \sqrt{D/m} \tag{4}$$

and for small differences in the location of the focal points, the spatial resolution of two focused solutes is

$$R_s \approx \frac{|x_{f,1} - x_{f,2}|}{2(\chi_1 + \chi_2)} = \frac{1}{4}\sqrt{\frac{1}{D\bar{m}^3}}\,|\,\bar{m}\Delta b + \Delta m\bar{b}\,| \tag{5}$$

where $\bar{m}$ and $\bar{b}$ are the averages of the slope and intercept, respectively, while Δm and Δb are differences in the slope and intercept. According to these formulas, narrow peaks are given by small dispersion coefficients and steep gradient slopes while higher resolution is provided by small dispersion coefficients and shallow slopes.

TYPE-2 ELECTROFOCUSING

As noted above, the earliest published example of type-2 electrofocusing where the applied gradient is in the hydrodynamic velocity was CACE as de-

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veloped by O'Farrell. A second example in this class is the SepStack apparatus invented by Culkin (22) and described by Mitchell (7) which electrofocuses proteins into a series of compartments separated by conventional membranes. The membranes are stacked parallel to one another with a thin gap between each pair, and a fixed electric current is passed through the entire stack (Fig. 2). A conductive liquid is introduced at the top of the stack and set to flow against the electrophoretic migration of proteins. A gradient in the flow is formed by extracting a portion of the crossflow from each compartment by means of a recirculating ultrafiltration (UF) or reverse osmosis (RO) membrane loop. Proteins then focus into those compartments in which the flow through the membrane most nearly counterbalances electromigration. A *linear* gradient is formed by removing fluid at the same rate from each compartment. Cooling is provided by a heat exchanger located on each UF/RO recirculation loop.

When operating the SepStack in batch mode, sample can be charged into any compartment(s) and focused into any other. In continuous mode, sample is introduced into a central compartment and is withdrawn from at least one compartment on each side of the feed. Scale up should proceed by increasing the membrane area, and resolution can be increased by introducing more compartments and implementing finer control of the UF/RO permeate streams. This is the first example of which I am aware of a segmented, type-2 electrofocusing column in which the gradient in each of the compartments can be externally controlled by the operator during a run. The benchtop prototype that

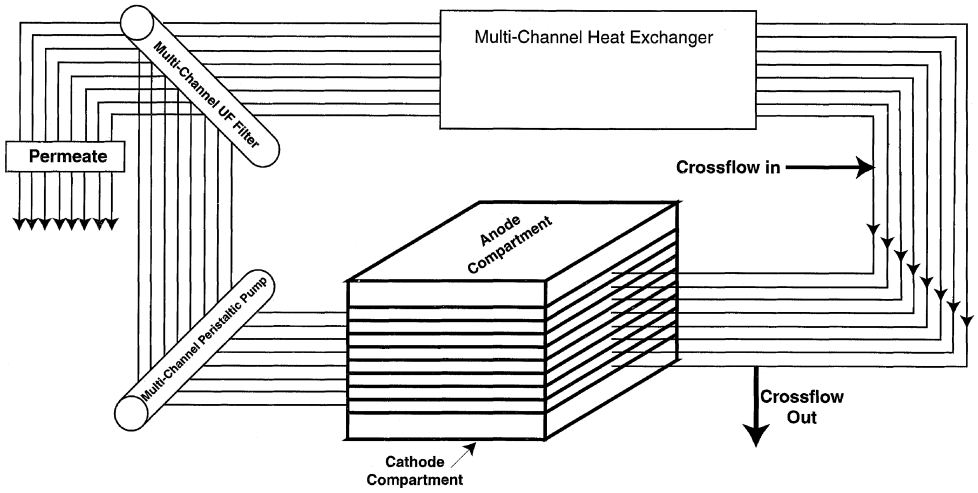

FIG. 2 Schematic of the SepStack, a membrane-based, type-2 electrofocusing device showing the arrangement of peripheral equipment around the membrane stack. The power supply furnishes an electric field which drives solutes against a gradient in the crossflow. The gradient is formed by withdrawing a small amount of running buffer through the UF modules mounted on each of the recycle loops.

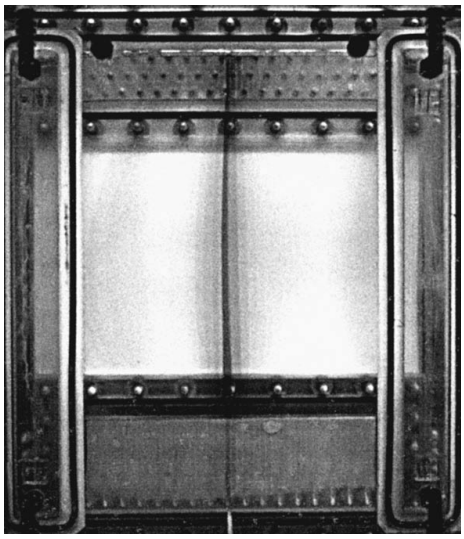

FIG. 3 Type-2 electrofocusing in a recycle zone electrophoresis chamber. In this case a jump in the crossflow velocity from left to right has been formed by mounting a UF module on the feed recycle loop and withdrawing a small amount of buffer through that membrane. The focused protein is bovine serum albumin which has been labeled with bromophenol blue to make it visible. The crescent-shaped blotch on the far left is bovine hemoglobin which was originally loaded in with the albumin but which excited the focusing window on the left because of its low electrophoretic mobility.

Culkin and Mitchell fabricated performed well in proof-of-concept experiments: a clean separation of model proteins was completed in a few minutes. However, the instrument was not developed further, apparently due to lack of interest on the part of investors.

A third type-2 electrofocusing device was demonstrated by Ivory (23) using a recycle zone electrophoresis apparatus that had been modified by placing a 10k MWCO UF filter on the feed-recycle loop. Withdrawing buffer as permeate through this membrane altered the crossflow so that proteins whose electrophoretic mobilities fell within the "focusing window" could collect near the feed port recycle loop while proteins outside this range would gradually migrate away from the feed loop (Fig. 3). This technique can be viewed as being similar in principle to Culkin's SepStack except that the membrane stack has been removed so focusing takes place in free solution at the velocity jump near the feed-recycle loop rather than in a membrane-bound compartment. And, while this approach has many of the advantages and disadvantages of Culkin's device, clearing model proteins from this chamber in practice took hours as compared with minutes in the SepStack. This was primarily due to 1) the relatively high volumetric holdup of the UF membrane on the feed-recycle loop and 2) the high protein concentration in the recycle UF loop, about 1% bovine serum albumin, which caused excessive fouling of the

membrane and unnecessary holdup of the contaminating protein, bovine hemoglobin.

TYPE-3 ELECTROFOCUSING

In this case a focusing gradient can be formed in the electric field by spatially varying the current density while maintaining a constant hydrodynamic counterflow. This technique was demonstrated by Koegler and Ivory (24) who showed that charged proteins could be separated and focused in an electric field gradient in a column packed with an SEC resin. A fluted cooling jacket was used to form a fixed linear gradient in the electric field which drove the proteins against a constant flow of buffer in a packed dialysis tube (Fig. 4). This instrument was slow, cumbersome to set up, and gave mediocre results, but it successfully illustrated an alternative focusing technique which was given the name electric field-gradient focusing (EFGF)

Following on the successful demonstration of Koegler's apparatus, an electrofocusing chamber was fabricated which used a linear array of 50 discrete electrodes rather than a fixed geometry to establish and maintain an electric

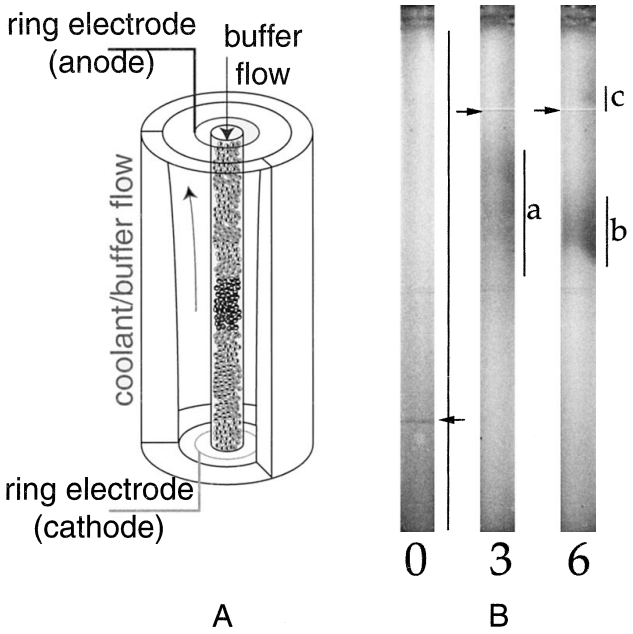

FIG. 4 Type-3 electrofocusing. (A) A linear electric field gradient is formed in this EFGF focusing device by using a fluted Plexiglas shell for the coolant/electrolyte flow. The solute is loaded into a cylindrical dialysis tube packed with an SEC resin and driven downward against the electric field by a flow of buffer. (B) Bovine hemoglobin (Sigma) is focused for 6 hours in the EFGF dialysis tube. At $t = 0$ the hemoglobin is spread uniformly over the tube. At $t = 3$ hours focusing is well underway but is not complete until 6 hours have passed.

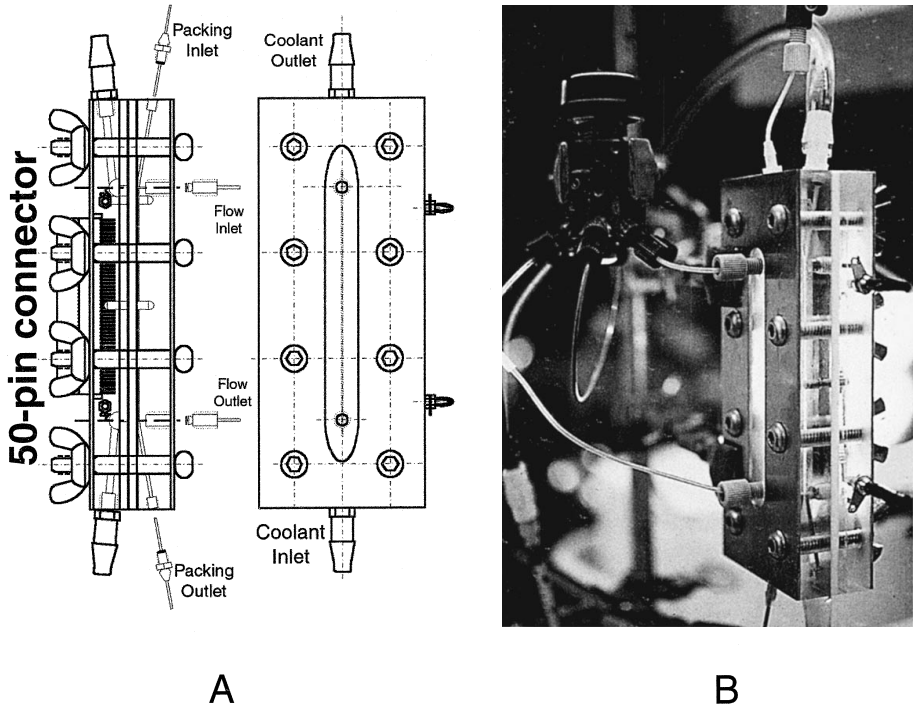

FIG. 5 (A) Front- and side-view drawings of the computer-controlled electrofocusing apparatus showing the flow, coolant, and packing inlets and outlets as well as the SCSI cable connector that links the electrodes to the controller. (B) The assembled device with sample loop injector and backlighting. During an experiment the focused bands are visible through the front window between the flow inlet and flow outlet ports.

field gradient (Fig. 5). Early versions of the controller first used zener diodes and, later, manually-adjustable potentiometers to control the voltage. However, the former was unstable to variations in the current and the latter was prone to drift, so control was eventually relegated to a digital computer. The advantage of this approach is that the voltages of the individual electrodes can be read by the computer and readily adjusted between or during a run by having the operator program in the new gradient profile via a Graphical User Interface written in C++. This instrument will be described further in the section of this paper entitled "Computer-Controlled Electrofocusing."

TYPE-4 ELECTROFOCUSING

In this case a gradient can be formed in the electric field by spatially varying the buffer conductivity using a simple salt, e.g., NaCl. As illustrated in Fig. 6, this is readily done by running two parallel buffer streams in cocurrent flow and in contact with each other via a dialysis membrane. If the buffer conductivities are mismatched between the two streams, transport of ions across the

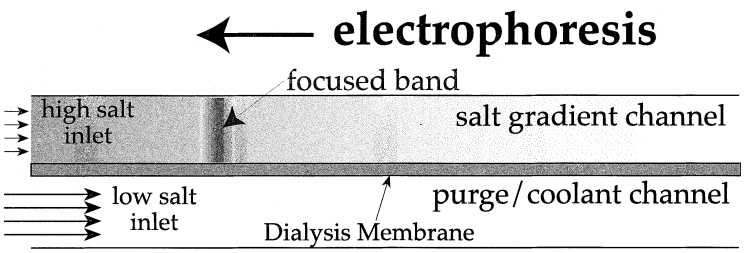

FIG. 6 Schematic of a Type-4 focusing device which uses a conductivity gradient to provide a gradient in the electric field. In this case, two cocurrent streams with different buffer compositions are allowed to interdiffuse through a dialysis membrane to form the conductivity gradient. Solutes then focus at that point in the gradient where their electrophoretic velocity is equal and opposite to their flow velocity on that side of the membrane.

dialysis membrane will form a conductivity gradient in each of the streams. Greenlee and Ivory (25) showed that proteins can then be focused in the electric field gradient formed by the conductivity gradient if it is opposed by a constant flow of buffer.

This apparatus was surprisingly simple to build and performed well despite its simplicity. A major drawback of this approach, however, was that it was difficult to find the right combination of buffer conductivities and flow rates to give adjustable, linear gradients. The two main reasons for this are that 1) the underlying diffusive technique used to provide the gradient is inherently exponential and 2) the movement of current-carrying ions across the membrane under the influence of the electric field tends to alter the gradient formed by diffusion.

TYPE-5 ELECTROFOCUSING

Although to my knowledge this has never been done, it is, according to Eq. (1), possible to focus a solute in a gradient which alters its effective frictional drag, N.B., which is inversely proportional to the solute's mobility, ω. One way this might be done is to use a chaotropic agent such as urea or an organic solvent like acetonitrile to partially unfold a protein population and then analyze that population using a reverse gradient to fractionate the unfolded forms. Assuming that fractionation is much faster than equilibration of the folded and unfolded states, a technology that will become available with the commercial introduction of millisecond microchip electrophoresis (26), it should be possible to fractionate out each of the intermediate folded forms and to follow their progress as they refold. This could be useful, for instance, in following the refolding of resolubilized proteins from inclusion bodies, incorrectly folded proteins eluted from reverse-phase columns, or in isolating proteins "trapped" in intermediate refolding conformations.

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COMPUTER-CONTROLLED ELECTROFOCUSING

While each of the techniques described earlier has its various strengths and weaknesses, Type-3 electro-focusing with an array of electrodes (27) offers several unique advantages over the other methods described in this paper. First, the electronically-generated field can take on arbitrary shapes including nonlinear profiles, step changes, and even locally reversed gradients, for example, to elute solutes. Second, the field shape can be monitored and maintained by computer to reduce noise and eliminate drift. Third, each electrode's potential can be modified manually at any time during a run, i.e., "on-the-fly," both in space and time to improve the resolution of bands. That is, during a run the operator can optimize the local properties of the field to tease proteins apart, sharpen an individual band, move a band to an offtake port, or set up a moving gradient to elute one or more bands from the chamber. In fact, with online, e.g., optical or potentiometric, monitoring in place, the operator can be assisted by a computer programmed to detect peaks and automatically adjust the field shape to optimize the separation and, when desired, to offload products. The ability to adjust the field gradient during an experiment in response to an ongoing fractionation led us to name this technique *dynamic field gradient focusing* (DFGF). This capability is illustrated in the separation described below.

DYNAMIC FOCUSING OF MYOGLOBIN

A 1-L solution of 10 mM Tris was titrated down to pH 8.8 with 1 M phosphoric acid and boiled to remove soluble gases. 10 mL of this solution was loaded into a syringe pump (Stoelting) and pumped through the packed section of the DFGF column overnight at 100 μL/h to equilibrate the column. 500 mL of this same buffer was cooled to about 7°C over an ice bath and recirculated over the backside of the packing membrane to remove Joule heat from the packed bed during a run (Fig. 7). Before starting the focusing experiment the packing was cleaned with two 10 μL pulses of 7 M urea.

2 mg of IEF-marker grade myoglobin (Sigma M9267) was dissolved in 2 mL of 10 mM Tris (unbuffered), then loaded into a 10 μL sample loop (Upchurch) attached to the DFGF column, and injected into the chamber. Power was then applied to the focusing column and controller at 400 V, and the electric field gradient was set to 6.9 V/cm^2. After approximately 15 minutes two myoglobin bands had formed, each about 0.2 mm thick and separated by about 0.2 mm (Fig. 8A). These bands were held in place for approximately 60 minutes before a second pulse of myoglobin was added to the column and the electric field gradient was dropped to 5.9 V/cm^2. (The run conditions are listed in Table 1.)

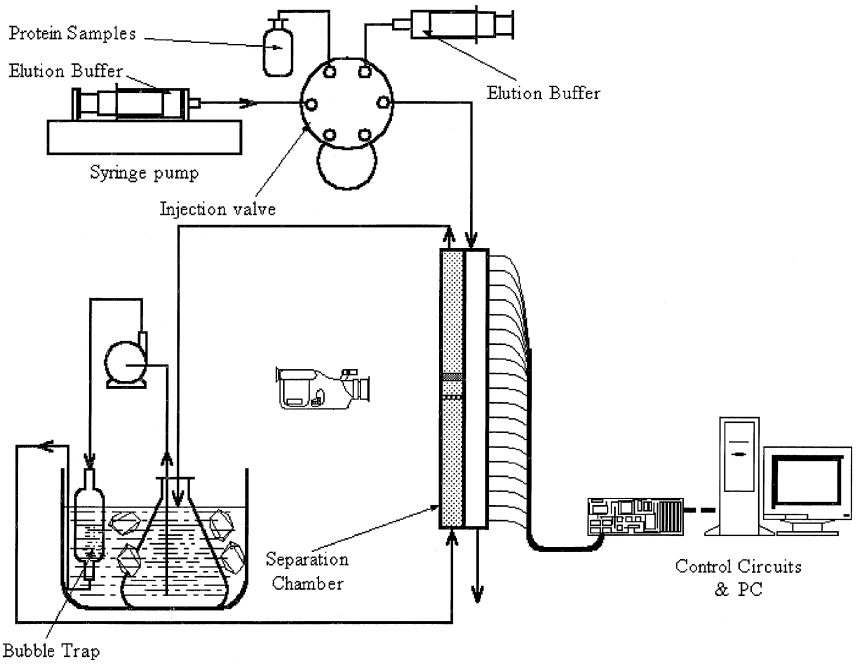

FIG. 7 Diagram of the DFGF apparatus showing peripheral equipment. In addition to its ability to alter the electric field profile from a computer, the composition of the recirculating buffer in the Erlenmeyer flask can be changed by adding electrolyte or titrating the pH to explore various separation conditions.

Over a period of about 30 minutes the two bands from the original pulse of myoglobin merged into a single band with the second pulse and then this split into the two bands shown in Fig. 8(B). As is evident in that figure, the resolution of the bands has noticeably improved. In the third part of this experiment the pH of the recirculating buffer was titrated down from 8.8 to 8.3 by adding phosphoric acid to the coolant reservoir. A third 10 μg pulse of myoglobin was then injected into the column and, as before, this merged with the myoglobin bands and then split into the two bands seen in Fig. 8(C). Again, the resolution of the two myoglobin bands noticeably improved as the pH was shifted closer to the pIs of the two myoglobin bands (~6.8, 7.2). As the pH of the coolant was titrated further to 8.0, the lower band flushed out of the chamber and the experiment was stopped. Figures 8(A–C) illustrate how separation conditions can be dynamically modified during a run to improve resolution either by altering the electric field gradient from the computer or by changing the properties of the buffer in the coolant reservoir.

One additional note: The myoglobin used in this experiment is intended to be used as a marker on an IEF-PAGE gel. It comes from the vendor freeze-dried and sealed in a small vial with 1.5 mg glycine added so that, after it is rehydrated, it will settle to the bottom of a PAGE sample well before power is

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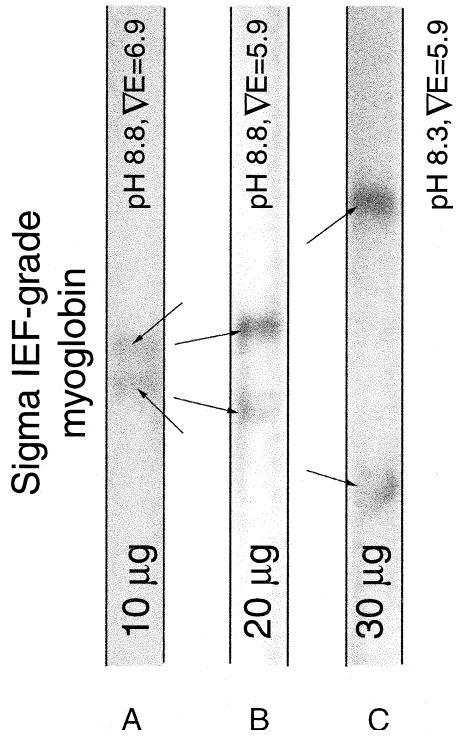

FIG. 8 Digitized 35 mm slides of focused Sigma IEF-grade myoglobin (MYO) in 10 mM Tris-phosphate buffer. (A) At pH 8.8, 400 V and $\nabla E = 6.9$, two bands approximately 0.5 mm thick are separated by about 0.5 mm. (B) Reducing the electric field gradient to $\nabla E = 5.9$ increases the resolution. (C) Reducing the pH in the coolant circuit to 8.3 by titrating the recirculating coolant with phosphoric acid further improves the band separation. Note that 10 μg of additional protein were added to (B) and another 10 μg to (C).

applied to the gel. No attempt was made to clear the glycine from the myoglobin sample before injecting it into the chamber since it is almost certain that the glycine dialyzes out of the packed section of the DFGF before the bands focus.

TABLE 1
Run Conditions for Proteins in Fig. 8
(10 mM Tris-phosphate buffer on 4.5 μm NovaPak-Diol)

	Protein(s)	pH	∇E (V/cm^2)	Flow (μL/h)	Applied voltage (V)	Protein mass loaded (μg)
A	Sigma IEF-marker myoglobin	8.8	6.9	100	400	10.0
B		8.8	5.9			20.0
C		8.3	5.9			30.0

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FUTURE WORK

Because resolution in the DFGF is inversely proportional to the square root of the electric field gradient as seen in Eq. (5), the resolving power of this instrument can be increased by improving the fidelity of the electronic controller circuitry, i.e., allowing it to produce shallower gradients. The existing controllers are able to regulate electrode voltages to within about 200 mV over an electrode spacing of 2.54 mm to give a minimum field gradient of 1.6 V/cm^2. A new controller is currently being tested which should be able to regulate the voltage to about 10 mV over 5.1 mm to give a minimum gradient of 0.02 V/cm^2. This will increase the resolution of DFGF by almost a factor of 9.

Another improvement will be to mount an optical detector on the chamber to provide real-time, in-situ monitoring of a separation as it is taking place and to feed that information back to the computer. This will allow the computer to detect peaks, locally adjust the field gradient to tease overlapping proteins apart, and then elute those peaks that are selected by the operator.

Next, it is important to implement a recovery strategy. There are several ways in which recovery could be carried out, the simplest of which is "field-gradient elution." In this approach a sample would first be fractionated into focused bands and then, to elute bands individually, the field-gradient profile would be sequentially shifted toward the chamber outlet one electrode at a time until each protein was eluted from the column. This strategy is similar to the type of gradient elution often practiced with adsorptive chromatography except that the "modulator" in this case is the electric field itself rather than a chemical gradient. As bands are brought to the end of the packed column, they are eluted into a 50-μm ID capillary similar to those used in capillary electrophoresis.

An alternative elution strategy would be to use multiple offtake ports located along the length of the packed column and, as each band in the load sample is identified and analyzed by the DFGF, to direct it to a separate offtake port. The advantage of this approach is that the computer can work with a number of different isolated bands at the same time, allowing for parallel processing of solutes. Also, a particularly refractory band could be sequestered away from the offtake ports until the column is cleared of other solutes. The full resolving power of the DFGF could be brought to bear on that refractory band at that time.

CONCLUSION

IEF is an excellent technique at analytical and preparative scales but alternative focusing techniques appear to have some important advantages for

electroprocessing at larger scales. Using the simple flux equation, this paper has shown that there are at least five classes of electrofocusing techniques of which IEF is only one. Furthermore, since all of these techniques share a common theoretical basis, they are all inherently capable of resolving solutes with the same power as IEF.

In one case, DFGF, the focusing gradient is an electric field established by an array of electrodes which are individually computer-controlled. The characteristic that sets DFGF apart from other purification techniques is its ability to refine a separation during a run by dynamically controlling the electric field gradient profile, a feature which is not available in conventional electrophoresis or chromatography. Finally, because DFGF separates molecules on the basis of their electrophoretic mobilities rather than by their charge or isoelectric point, it provides a purification strategy which is orthogonal both to isoelectric focusing and to the various forms of chromatography, including ion exchange.

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NOTATION

b	gradient intercept
c	protein concentration
D	protein diffusivity
I_x	axial current density
M_T	total mass in focusing column
m	gradient slop
N_x	molar flux of protein
R	resolution
u_x	apparent chromatographic velocity
t	packed column thickness
w	packed column width
x	axial coordinate
x_f	axial coordinate
z	protein charge

Greek Symbols

χ	peak standard deviation
σ	electrical conductivity
ω	absolute mobility

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