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Preparative-scale isoelectric trapping separations using a modified Gradiflow unit

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Abstract

The Gradiflow BF200 preparative electrophoretic unit (Gradipore), which has been developed for size-based and charge-sign-based protein separations and in which the hydraulic flow path of the recirculating sample stream in the separation cartridge is orthogonal to the electric field, has been modified to carry out binary protein separations using the principles of isoelectric trapping. The disposable separation cartridge contained three isoelectric membranes which, along with the cartridge holder, formed the anode and cathode compartments and the anodic and cathodic separation compartments. The utility of the modified instrument was demonstrated by effecting a binary separation of chicken egg white across an isoelectric point 5.5 isoelectric membrane. The desalting and subsequent binary separation steps proved to be remarkably rapid, due to the favorable combination of short electrophoretic path, high electric field strength and large effective isoelectric membrane surface area.

Keywords: Isoelectric focusing; Preparative electrophoresis; Isoelectric trapping; Membranes; Gradiflow; Proteins

1. Introduction

As indicated by the annual recurrence of multiple reviews, interest in all forms of preparative-scale, non-gel, electrophoretic separations has grown steadily over the last 7 years [1–12]. Success of a proposed preparative electrophoretic separation method depends not only on its ability to create adequate separation selectivity (by zone electrophoresis, isotachophoresis or isoelectric focusing), but also on its ability to balance the opposing requirements that favor high resolution and high production rates, respectively. This requires means to effectively dissipate Joule-heat and keep convective mixing to a minimum.

When the target compounds are ampholytic, such as proteins, isoelectric focusing (IEF) in carrier ampholytes or low conductivity Bier's buffers [13] is often favored over zone electrophoresis, because the focusing mechanism at least partially offsets the undesirable effects of diffusion and convective mixing [3–6]. Isoelectric trapping (IET), an alternative to recirculating IEF [3] or free-flow IEF [4,5], achieves separation of proteins by trapping them in pure, isoelectric form [7,8] in compartments formed by immobilized isoelectric gel segments [14–17] or isoelectric membranes [18–34] whose isoelectric points (p*I* values) bracket the p*I* values of the

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proteins. In the commercial implementation of the multicompartmental electrolyzer [35], the proteins to be separated are loaded into (or flow through) the 1 cm wide separation channels, creating orthogonal hydraulic and electrophoretic transport paths for the proteins. This arrangement facilitates external heat removal (by cooling the sample reservoirs) and permits the use of large sample feed volumes [18–35]. In addition to the purification of large biomolecules, the commercial multicompartmental electrolyzer, the Isoprime [35], has also been used for the separation of small molecules, such as the enantiomers of dansyl phenylalanine in the presence of 30 m*M* hydroxypropyl-β-cyclodextrin, and afforded a production rate of about 0.1 mg/h [36].

The Gradiflow technology [37–46] combines hydraulic flow of the protein mixture through two shallow separation channels with orthogonal electrophoretic transport of the proteins across a single separation membrane that isolates the two separation channels. Selective transport across the separation membrane can be realized when the hydrated ion radii of the like-charged proteins are smaller and larger, respectively, than the average pore size of the separation membrane that has a narrow pore-size distribution (size-based separations). Selective transport across the separation membrane can also be achieved when the charge-sign of the proteins differ: cationic proteins pass through the wide-pore separation membrane when loaded into the anodic separation compartment, while anionic proteins remain in the anodic separation compartment (charge-sign-based separations). Separation selectivity can be easily tuned by changing the average pore size of the separation membrane (for size-based separations) or by changing the pH of the background electrolyte (BGE) with respect to the p*I* values of the proteins to be separated from each other (for charge-sign-based separations). High resolution is achieved by repeating the binary separation one more time under slightly different conditions. High throughput is achieved by combining shallow separation channels and thin membranes (keeping the electrophoretic migration distances to a minimum and the electric field strengths to a maximum, even at low applied potentials) with large separation membrane surface areas and effective Joule-heat removal. In order to offer long operation times, yet minimize the volume of BGE used for the separation, the anolyte and catholyte streams are recirculated: they originate from and return back into the same BGE reservoir. By suitable choice of the BGE components, pH changes in the BGE brought about by the anode and cathode reactions offset each other maintaining the original pH of the BGE.

The current, commercial implementation of the Gradiflow technology (BF200 unit) has two limitations that originate from the single BGE reservoir approach. The first limitation is that salt that is removed from the sample stream during its initial passage through the separation compartment is returned, albeit diluted, as the BGE is recirculated. Thus, reduction of the salt concentration in the sample requires either the use of large volumes of BGE to increase the degree of salt dilution or the replacement of the BGE after the initial salt dilution step. The second limitation of the BF200 unit is that electrophoretic separations that require the use of a nonhomogeneous BGE system, such as ITP or IEF, cannot be conducted on the BF200 unit. The objective of the present paper is to describe a modification of the BF200 unit that facilitates its use for effective desalting, as well as IET separations.

2. Experimental

2.1. Chemicals

Phosphoric acid, sodium hydroxide, triethanolamine, acetic acid and hydroxypropylmethylcellulose (HPMC, average molecular mass 86 000), acrylamide, *N,N′*-methylene bis-acrylamide, ammonium persulfate and tetramethylethylenediamine were obtained from Aldrich (Milwaukee, MI, USA), while carrier ampholytes Pharmalyte pH 3–10, terbutaline and tyramine were from Sigma (St. Louis, MO, USA). The piperidinium salt of dansyl-phenylalanine (Dns-Phe) was purchased from NBS Biological (Huntingdon, UK). The Immobiline chemicals were from Fluka (Ronkonkoma, NY, USA).

All solutions were freshly prepared using deionized water from a Milli-Q unit (Millipore, Milford, MA, USA). In the full-column imaging capillary isoelectric focusing (cIEF) separations, 80 m*M* phosphoric acid and 100 m*M* sodium hydroxide were

used as anolyte and catholyte, respectively. Both solutions contained 0.17% (m/v) HPMC. The carrier ampholyte stock solution contained 8% (v/v) carrier ampholytes (p*I* 3–10) and 0.02 m*M* Dns-Phe, 0.03 m*M* terbutaline and 0.03 m*M* tyramine as p*I* markers (approximate p*I* values 3.5, 9.6 and 10.0, respectively). The samples collected during the preparative IET separations were mixed with this stock solution at a rate of 50 μl to 150 μl. For the preparative-scale IET separations, the anolyte was 2 m*M* acetic acid, the catholyte was 1 m*M* triethanolamine. The sample was chicken egg white, diluted in deionized water at a rate of 1 to 25.

2.2. Analytical cIEF equipment

The analytical scale, full-column imaging IEF separations were carried out on an iCE280 unit (Convergent Biosciences, Toronto, Canada) with its imaging detector operated at 280 nm. Samples were injected into the iCE280 by an Alcott 718AL autoinjector using the 96-well microtiter plate adapter (Alcott, Norcross, GA, USA). The cIEF separations were obtained on a 5 cm×100 μm I.D., fluorocarbon-coated fused-silica capillary (Convergent Biosciences) with an applied potential of 3000 V, transfer time of 1.1 min and focusing time of 5 min. The acquired cIEF images were processed by the EZ Chrom software (Scientific Software, Pleasanton, CA, USA).

2.3. Preparative IET equipment

All preparative-scale IET separations were completed with a modified BF200 unit (Gradipore, French's Forest, Australia). The heart of the system is the disposable separation cartridge that is shown in its exploded view in Fig. 1, and its cross-sectional assembly view in Fig. 2. The separation cartridge consists of a housing, an anodic gasket with inlet and outlet holes, an anodic isoelectric membrane with inlet and outlet holes, an anodic separation compartment grid with inlet and outlet holes, an isoelectric separation membrane, a cathodic separation compartment grid with inlet and outlet holes, a cathodic isoelectric membrane with inlet and outlet holes, a cathodic gasket with inlet and outlet holes, and a retaining clip. The active surface area of the isoelectric membranes is about 15 cm^2. The gaskets are 1 mm thick, while the separation compartment grids are 1.2 mm thick. The isoelectric membranes are about 0.15 mm thick. The anode and cathode, made of platinum-coated expanded titanium mesh, are located in the movable anode and cathode compart-

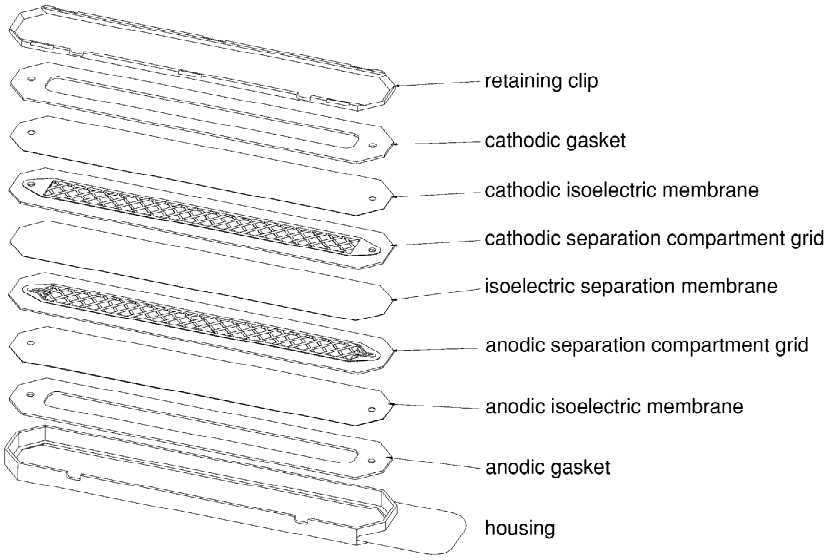

Fig. 1. Exploded view of the disposable separation cartridge of the modified Gradiflow BF200 preparative electrophoretic unit.

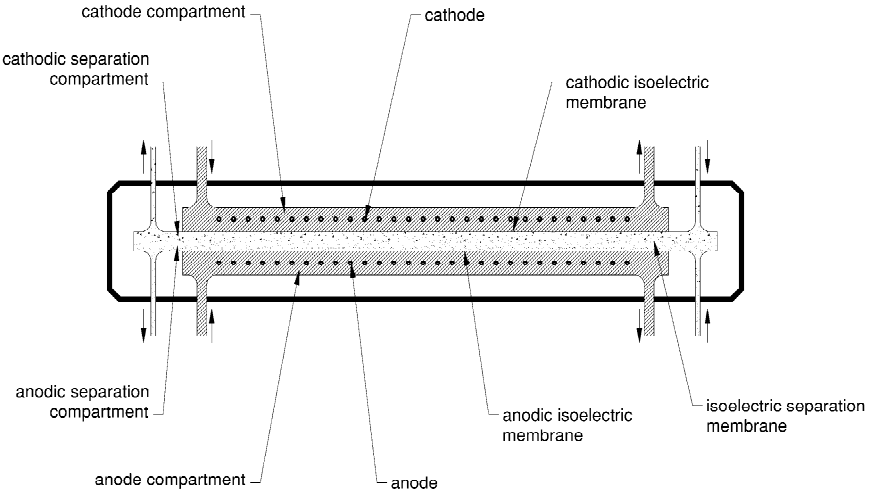

Fig. 2. Cross-sectional assembly view of the disposable separation cartridge of the modified Gradiflow BF200 preparative electrophoretic unit.

ments of the BF200 unit, positioned across and about 2 mm away from the surface of the anodic and cathodic gaskets. The anode and cathode compartments are pressed against the anodic and cathodic gaskets of the cartridge to form four scaled compartments through which the anolyte, the two sample streams and the catholyte flow.

The schematic of the entire unit is shown in Fig. 3. The anolyte and catholyte containers hold ice-filled, 4 l stainless steel beakers which cool the anolyte and catholyte. The anolyte and catholyte are fed to the anode and cathode compartments at a flow-rate of 2 l/min by independent centrifugal pumps. The high flow-rate provides for efficient convective removal of Joule-heat from the separation compartment. Two peristaltic pumps recirculate the sample streams at a flow-rate of 20 ml/min through the anodic and cathodic separation compartments and the stainless steel, flow-through heat exchangers immersed into the anolyte container. A 250 V, 1 A d.c. power supply (E-C Apparatus, Holbrook, NY, USA) is connected to the anode and cathode through safety switches which automatically disconnect the power supply from the separation unit when the covers of either the anolyte or catholyte containers are opened.

The isoelectric membranes were prepared from Immobiline chemicals, acrylamide, *N*,*N*′-methylene bis-acrylamide, ammonium persulfate and tetramethylethylenediamine as described in Chapters 4 and 5 of Ref. [35]. The isoelectric membranes were supported by a 0.1 mm thick polyethylene terephthalate fabric (Mitsui, Melbourne, Australia).

2.4. Preparative IET separations

Prior to a preparative IET separation, the separation cartridge was assembled using three isoelectric membranes: a p*I* 4.0 anodic membrane, a p*I* 5.5 separation membrane and a p*I* 7.0 cathodic membrane. Next, the cartridge was installed into the modified BF200 unit, the anolyte and catholyte containers and the sample containers were filled with deionized water, the pumps were turned on and operated for half an hour to check for leaks. Then, the anolyte container was filled with 1 l of 2 m*M* acetic acid solution, the catholyte container was filled with 1 l of 1 m*M* triethanolamine solution, and the stainless steel beakers were filled with crushed ice to cool the anolyte and catholyte to the operating temperature. The sample containers were filled with 10 ml each of a chicken egg white solution (chicken egg white diluted in deionized water at a ratio of 1 to 25). Then, the pumps and the power supply were turned on and 0.5 ml samples were taken from each stream at every 15 min. The pH of the samples was measured with a solid-state microelectrode, pH16-SS, and a Model IQ240 pH meter (IQ, San Diego, CA, USA). Their protein composition was analyzed

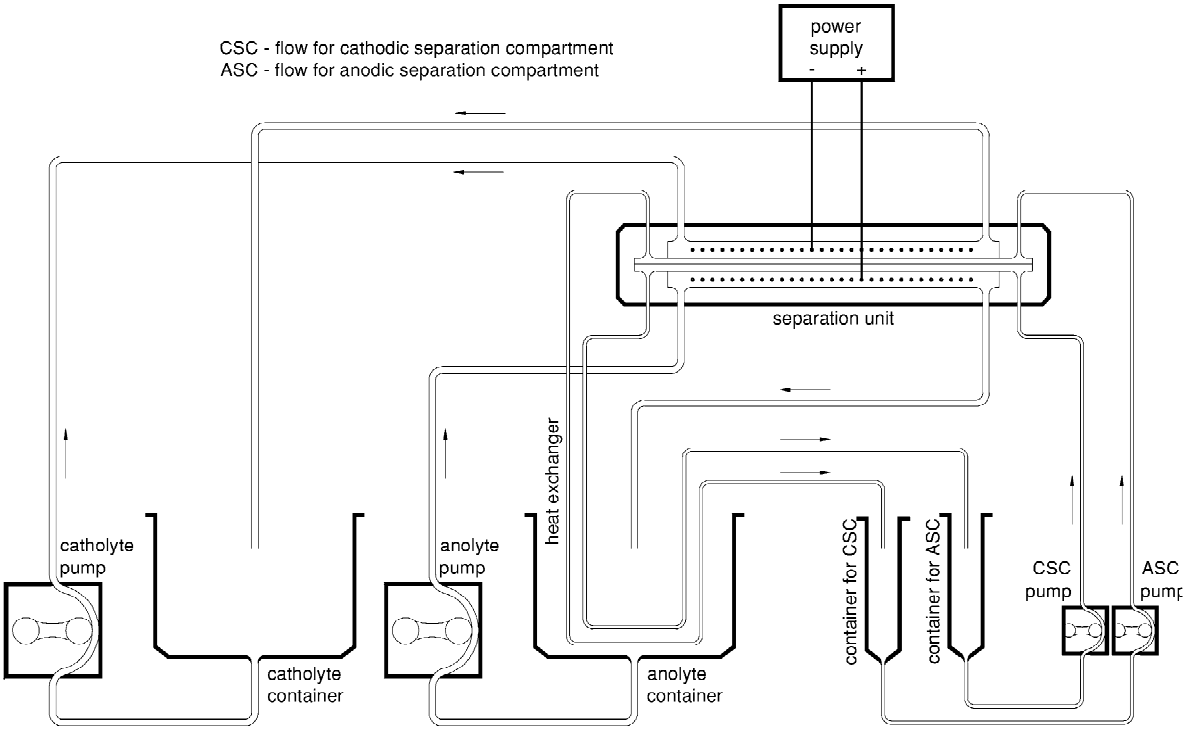

Fig. 3. Schematic of the Gradiflow BF200 unit modified for preparative isoelectric trapping separations.

by the iCE280 full column imaging cIEF unit. Once the separation was deemed complete, the potential and the pumps were turned off and the separated proteins were harvested. The cartridge was removed and the system was sanitized with a 1 *M* NaOH solution.

3. Results and discussion

Fig. 4 shows the results for the binary IET separation of the chicken egg-white feed sample in the modified BF200 IET unit. At the beginning of the separation, 10 ml of the chicken egg white solution was loaded into each of the anodic and cathodic separation compartments. These solutions were recirculated through the respective separation compartments while a separation potential of 250 V was applied. The initial current was 130 mA; its value dropped to 6 mA in 4 min and remained constant until the end of the separation. The duration of the experiment, 15 min, was determined by the

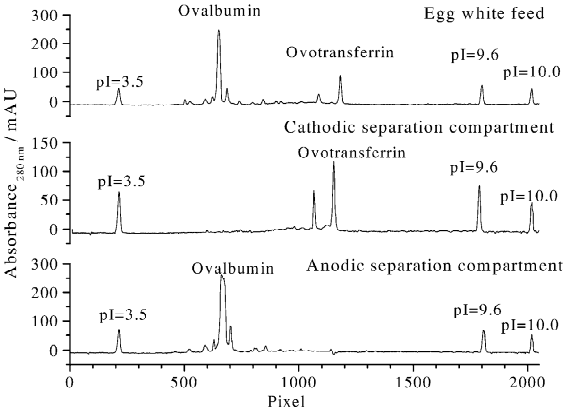

Fig. 4. Full-column imaging cIEF separation of the chicken egg white feed sample (top panel), aliquot of the content of the cathodic separation compartment after 15 min of electrophoresis (middle panel) and aliquot of the content of the anodic separation compartment after 15 min of electrophoresis (bottom panel). For experimental details, see Results and discussion.

rapid decrease in the volume of the liquid in both the anodic and cathodic sample reservoirs, indicating the presence of a strong, undesirable bulk flow. No proteins with p*I* values in the 4 to 7 range were found either in the catholyte or the anolyte at the end of the separation (electropherograms not shown), indicating that the anodic and cathodic isoelectric membranes indeed managed to trap the p*I* 4–7 proteins in the separation unit.

The top panel of Fig. 4 shows the CIEF separation of the chicken egg white feed sample obtained with the iCE280 instrument. In the iCE280 instrument, the UV absorbance profile of the 5 cm long separation capillary is imaged onto 2043 pixels of the detector. Therefore, the pH gradient in the capillary spans the 1 to 2043 pixel range and the position of the protein bands in the pH gradient can be described in terms of pixels. In order to relate the band position expressed in pixels to the pH value, three p*I* markers, Dns-Phe (approximate p*I*=3.5), terbutaline (approximate p*I*= 9.6) and tyramine (approximate p*I*=10.0) were added to each sample and identified in the electropherograms. The main components of chicken egg white are ovalbumin (largest peak between pixels 600 and 750, approximate p*I* value 4.6) and ovotransferrin (largest peak between pixels 1050 and 1250, approximate p*I* value 6.2). Lysozyme and avidin, which have p*I* values above 10, are not observed in the imaged portion of the capillary with the p*I* 3–10 carrier ampholytes.

The middle panel in Fig. 4 shows the cIEF separation of the aliquot collected from the cathodic separation compartment after 15 min of electrophoresis. The pH of the solution in the cathodic separation compartment at the end of the separation was 6.6. Proteins with p*I* values lower than 5.5 were not found in this stream.

The bottom panel in Fig. 4 shows the cIEF separation of the aliquot collected from the anodic separation compartment after 15 min of electrophoresis. The pH of the solution in the anodic separation compartment at the end of the separation was 4.7. Proteins with p*I* values higher than 5.5 were not found in this stream.

What is remarkable about this binary separation is its speed: despite the high initial salt content of the sample, all p*I* 4–5.5 and p*I* 5.5–7 proteins present in 0.8 ml of fresh chicken egg white were completely moved into the anodic and cathodic separation compartments, respectively, in as little as 15 min. In salt-free samples, the separation speed is expected to be higher and the power consumption is expected to be lower. Further work is underway in our laboratories to reduce the magnitude of the observed untoward bulk flow during the IET separation and further improve the speed of the separation.

4. Conclusions

This paper has shown that the Gradiflow BF200 preparative electrophoretic unit, in which the recirculating hydraulic flow path of the sample stream in the disposable separation cartridge is orthogonal to the electric field, can be modified to carry out rapid, successful, binary protein separations using the principles of isoelectric trapping implemented with the help of three isoelectric membranes. The demonstrated rapid separation speed is a result of the favorable combination of short electrophoretic path, high electric field strength and large effective isoelectric membrane surface area.

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