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Preparative-scale isoelectric trapping enantiomer separations

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Abstract

The new Gradiflow BF200 IET unit, developed for isoelectric trapping protein separations has been modified and used to carry out preparative-scale enantiomer separations. Hydroxypropyl β-cyclodextrin was used as the chiral resolving agent to induce an isoelectric point difference between the enantiomers. Three isoelectric membranes with isoelectric points below, in between and above the isoelectric points of the complexed enantiomers were used to trap the separated enantiomers in the anodic and cathodic separation compartments of the Gradiflow BF200 IET apparatus, respectively. The production rates were about 15 times higher than those previously obtained with another isoelectric trapping device and about 30% higher than those obtained in a continuous free-flow electrophoretic device operated in the isoelectric focusing mode. The remarkable separation speed observed in the modified Gradiflow BF200 IET unit is attributed to the favorable interplay of the short electrophoretic transfer distance, the high electric field strength and the large effective surface areas of the isoelectric membranes.

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1. Introduction

The original Gradiflow binary protein separation technology combines the hydraulic flow of a protein mixture through shallow anodic and cathodic separation compartments with the selective, orthogonal, electrophoretic transport of some of the proteins of the mixture across a polyacrylamide separation membrane located between the separation compartments [1–10]. In addition to facilitating selective protein transport, the polyacrylamide membrane also inhibits convective mixing of the contents of the separation compartments. Polyacrylamide restriction membranes whose pores are smaller than the size of the target proteins prevent the migration of the target proteins out from the separation compartments and into the electrode compartments. Proteins are selectively accumulated in the respective separation compartments by varying either the pore size of the separation membrane (size-based separations) or the pH of the background electrolyte (charge sign-based separations). In the latter approach, the pH of the selected buffer forces some of the proteins to become negatively charged, the others positively charged, and thus cause them to move into the anodic or cathodic separation compartment, respectively. In the original Gradiflow technology, the anolyte, background electrolyte and catholyte all have the same composition. By using a common electrolyte reservoir for the recirculated anolyte and catholyte, compositional integrity of the electrolyte solution is maintained for long periods of time because pH

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changes brought about by the anodic and cathodic electrode reactions offset each other.

Righetti and co-workers [11–33] introduced an ingenious alternative electrophoretic protein separation method, the isoelectric trapping (IET) method. In isoelectric trapping, isoelectric membranes of different isoelectric point (pI) values are used to form separation compartments in a multicompartmental electrolyzer. The target proteins are trapped in the compartments in their isoelectric state and are recovered in pure form, free of background electrolyte components.

Isoelectric focusing (IEF) enantiomer separations were first reported by Righetti et al. [34] who saturated a polyacrylamide slab gel with a mixture of β-cyclodextrin (CD) and carrier ampholytes. Glukhovskiy and Vigh [35] developed an analytical expression to predict the magnitude of the isoelectric point difference (ΔpI) that can be generated between the enantiomers by a noncharged chiral resolving agent such as a noncharged CD:

$$\Delta pI'_{R,S} = \frac{1}{2} \cdot \log\left(\frac{1 + K_{S^-CD}[CD]}{1 + K_{R^-CD}[CD]} \frac{1 + K_{H^+RHCD}[CD]}{1 + K_{H^+SHCD}[CD]} \right) \quad (1)$$

where K_{R^-CD}, K_{S^-CD}, K_{H^+RHCD}, and K_{H^+SHCD} are the equilibrium constants for the formation of the anionic complexes of the two enantiomers (R^-CD and S^-CD) and the cationic complexes of the two enantiomers (H^+RHCD and H^+SHCD), and [CD] is the species concentration of the free, noncharged cyclodextrin. This relationship has proved applicable both for the capillary IEF separation [35–37] and preparative-scale continuous free-flow IEF separation of enantiomers [35,38]. It also formed the basis for the preparative-scale separation of the enantiomers of dansyl phenylalanine in the commercial version of the multicompartmental electrolyzer, the Isoprime unit [39], by isoelectric trapping in the presence of 30 mM hydroxypropyl β-cyclodextrin and resulted in a production rate of about 0.1 mg/h [40].

Recently, Ogle et al. [41] described the design of a new IET apparatus derived from the Gradiflow BF200 unit that has extended the capabilities of the Gradiflow binary protein separation technology to carry out electrophoretic separations, including isoelectric trapping separations, that require the use of non-homogeneous background electrolyte systems.

The objective of the present paper is to (i) describe modifications to the separation unit of the new the Gradiflow BF200 IET apparatus that facilitate more efficient IET separations, (ii) show that the apparatus is suitable for the IET separation of enantiomers, and (iii) show that the apparatus can provide production rates that compare favorably with those achieved both in the Isoprime unit [40] and the continuous, free-flow electrophoretic system, the Octopus, operated in isoelectric focusing mode [35,38,42].

2. Experimental

2.1. Chemicals

Phosphoric acid, sodium hydroxide, ethanolamine and hydroxypropylmethylcellulose (HPMC, average molecular mass 86 000) were obtained from Aldrich (Milwaukee, MI, USA), while carrier ampholytes Pharmalyte pI 2.5–5 were from Sigma (St. Louis, MO, USA). The racemic mixture of the piperidinium salt of dansyl-tryptophan (Dns-Trp) was obtained from NBS Biological (Huntingdon, UK). Hydroxypropyl-β-CD (HP-β-CD) with an average degree of substitution of 4.7 was purchased from Cerestar (Hammond, IN, USA) for use as the chiral resolving agent in the preparative-scale separations. All solutions were freshly prepared using deionized water from a Milli-Q unit (Millipore, Milford, MA, USA).

For the preparative-scale IET separations, the anolyte was 2 mM phosphoric acid, the catholyte was 2 mM ethanolamine. The sample was a racemic mixture of 0.75 mM Dns-Trp dissolved in 60 mM HP-β-CD in deionized water.

The samples collected in the IET separation were analyzed by full-column imaging capillary isoelectric focusing (cIEF) as described in Ref. [38]. The anolyte and catholyte were 80 mM phosphoric acid and 100 mM sodium hydroxide, respectively, and both solutions contained 0.17% (m/v) HPMC to minimize the electroosmotic flow. The carrier ampholyte stock solution contained 3.2% v/v carrier ampholytes (pI 2.5–5), 60 mM HP-β-CD and 0.17% (m/v) HPMC. This solution was used to dilute the samples collected from the IET separation at a rate

of 4–10 μl sample to 196–190 μl of the carrier ampholyte stock solution.

2.2. Analytical equipment

Full-column imaging cIEF separations were carried out on an iCE280 unit (Convergent Biosciences, Toronto, Canada). The imaging detector was operated at 280 nm. The instrument was connected to an Alcott 718AL autoinjector that was equipped with a 96-well microtiter plate adapter (Alcott, Norcross, GA, USA). The separation cartridge contained a 5-cm×100 μm I.D., fluorocarbon-coated fused-silica capillary (Convergent Biosciences). Separations were obtained at 3 kV, with a transfer time of 2.5 min and a focusing time of 8.5 min [38]. The cIEF images were processed by the EZ Chrom software (Scientific Software, Pleasanton, CA, USA).

The pH of the collected fractions was measured with a solid state microelectrode, pH16-SS, and a Model IQ240 pH meter (IQ, San Diego, CA, USA).

2.3. Preparative equipment

All preparative-scale IET separations were completed with a BF200 IET unit (Gradipore, French's Forest, Australia). The design of the system was described in detail in Ref. [41]. In order to mitigate cross-membrane bulk flow that was observed previously [41], the outlet connections in both the separation compartments and the electrode compartments have been widened to make their cross sectional area equal to the flow-through cross sectional area of the respective compartments and reduce their flow resistance. The separation compartment has also been redesigned: the lattice [41] which caused cross-channel mixing parallel to the direction of the electric field and reduced the separation efficiency has been eliminated. The depth of both separation compartments has been reduced from 1250 to 500 μm, more than halving the distance the analytes have to migrate electrophoretically. The length of the separation compartment is 135 mm, its width is 11.5 mm, which results in an active transfer surface area of 15.525 cm^2 and compartment volume of 0.776 ml.

The disposable separation cartridge is still assembled the old way [41]: it contains the 1000 μm thick silicon rubber anodic gasket, the pI=3 anodic isoelectric membrane, the 500 μm deep anodic separation compartment, the pI=3.7 isoelectric separation membrane, the 500-μm thick cathodic separation compartment, the pI=7.5 cathodic isoelectric membrane, and the 1000-μm thick silicon rubber cathodic gasket, all from Gradipore. The useful surface area of the isoelectric membranes is about 15 cm^2, their thickness is about 0.15 mm. The electrodes, housed in the separation unit are made of platinum-coated expanded titanium mesh and are located across, and about 1.5 mm away from the surface of the anodic and cathodic gaskets. The anode compartment, the anodic separation compartment, the cathodic separation compartment and the cathode compartment are formed when the separation unit is closed.

The total anode-to-cathode distance in the redesigned separation unit has been reduced from the original 8.5 to 6.45 mm. This change has increased the effective electric field strength that can be achieved for the same applied potential. This, together with the shorter electrophoretic migration distances is expected to increase the speed of the IET separation.

The ice-cooled anolyte and catholyte are recirculated at a flow-rate of 2 L/min, the ice-cooled sample streams are recirculated at a flow-rate of 10 ml/min. A 900-V, 1200-mA d.c. power supply (E-C Apparatus, Holbrook, NY, USA), connected through safety switches provides the separation potential.

3. Results and discussion

3.1. Preparative IET separations

First, the cartridge was assembled, placed into the separation unit, all reservoirs were filled with deionized water and the recirculating pumps were turned on to check the integrity of the seals. The liquid volumes in each reservoir remained constant during the seal test, indicating that the flow resistance reductions achieved by the enlarged exit ports eliminated the undesirable, pressure-induced cross-membrane flows that were observed earlier [41]. Next, the anolyte container of the BF200 IET apparatus was filled with 250 ml of 2 mM phosphoric acid solution

and the catholyte container was filled with 250 ml of 2 m*M* ethanolamine solution.

Then, crushed ice was loaded into the system to cool the anolyte and catholyte to the operating temperature. The sample containers were filled with 50 ml each of 0.75 m*M* racemic Dns-Trp dissolved in 60 m*M* HP-β-CD solution. Only a small fraction of the enantiomers (1.55% m/m) is in the electric field at any given moment, because the volume of the separation compartment is 0.776 ml and convenient sample volumes are in the 50-ml range. This results in an initial duty cycle of 0.776 ml/50 ml = 0.0155.

Next, the power supply was set at a constant power of 10 W and turned on. A rapid rise in the separation potential indicated that the piperidinium counterion of racemic Dns-Trp was completely removed in as short a time as about 15 min. Aliquots of 0.5 ml were taken from each recirculating stream every 60 min and analyzed for enantiomeric purity by the iCE280 full column imaging cIEF unit. Once the targeted enantiomeric purity (95%) was achieved in the anodic separation compartment (after 9 h of separation), the potential and the pumps were turned off and the enantiomers were harvested. The separation cartridge was disassembled and the system was cleaned with a 1 *M* NaOH solution. No enantiomers were found either in the catholyte or the anolyte at the end of the separation.

Fig. 1 shows the change in the enantiomer composition (expressed as %, m/m, enantiomeric purity) in the anodic and cathodic sample reservoirs as a function of time. Fig. 2 shows the full column imaging cIEF separation of the contents of the anodic and cathodic sample reservoirs after 9 h of electrophoresis. The iCE280 instrument yields electropherograms in which absorbance is shown as a function of the position (expressed in pixels) of the analyte band on the capillary. Since the pH gradient runs from pH 2.5 to 5 from the beginning of the capillary (1st pixel) to its end (2043rd pixel), band position in the capillary is correlated with the local pH of the focused carrier ampholyte solution.

The enantiomeric purity increased with time in both reservoirs, the rate of increase slowed down as the separation progressed. The final concentrations and enantiomeric purities were slightly different in the anodic and cathodic sample reservoirs. The

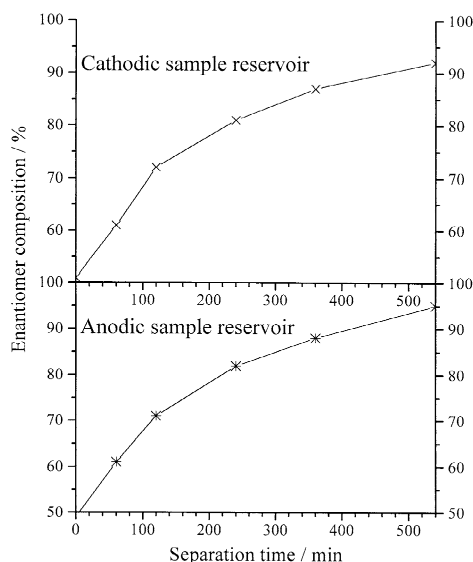

Fig. 1. Enantiomer composition (expressed as %, m/m, enantiomer purity) in the anodic and cathodic sample reservoirs as a function of separation time during the preparative-scale IET separation of the enantiomers of Dns-Trp in the modified Gradiflow BF200 IET unit. Isoelectric membranes: p*I* = 3.0 (anodic), p*I* = 3.7 (separation), p*I* = 7.5 (cathodic). Sample feed rate: 10 ml/min. IET power: 10 W.

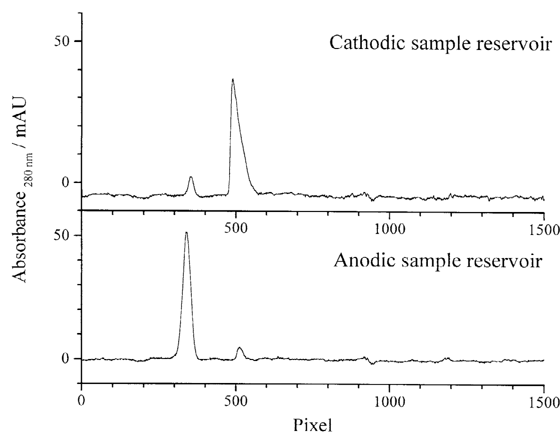

Fig. 2. Full-column imaging cIEF separation of the aliquots collected from cathodic separation compartment (top panel) and anodic separation compartment (bottom panel) of the modified Gradiflow BF200 IET unit after 540 min of electrophoresis of the racemic Dns-Trp feed mixture. Isoelectric membranes: p*I* = 3.0 (anodic), p*I* = 3.7 (separation), p*I* = 7.5 (cathodic). Sample feed rate: 10 ml/min. IET power: 10 W.

solution in the cathodic sample reservoir was more dilute than in the anodic sample reservoir and the enantiomeric purity was lower (92%) than the targeted enantiomer purity in the anodic sample reservoir (95%). The reason for this behavior is that after about 180 min of electrophoresis, the volume of liquid in the cathodic sample reservoir began to increase, indicating that the p*I* = 7.5 membrane exposed to the basic catholyte became more permeable. This decreased the concentration of the enantiomers in the cathodic separation compartment, consequently decreased the effective time the enantiomers present in the cathodic sample stream spent in the electric field (i.e., it lowered the duty cycle in the cathodic sample stream). Obviously, this reduced the amount of the lower p*I* enantiomer complex that was removed from the cathodic sample reservoir compared to the amount of the higher p*I* enantiomer complex that was removed from the anodic sample reservoir.

In order to be able to compare the production rates with earlier, published data obtained with two different preparative electrophoretic units, the separation was stopped when the enantiomeric purity in the anodic sample stream reached the 95% level, the approximate average purity level we obtained with the Isoprime unit [40] and the Octopus continuous free-flow electrophoretic device [38]. After pooling all the respective fractions, the production rates were 0.1 mg Dns-Phe/h with the Isoprime unit [40], 1.2 mg Dns-Trp/h with the Octopus unit [38], and 1.8 mg Dns-Trp/h with the Gradiflow BF 200 IET unit. The direct electrophoretic energy consumption values were about 70 W h/mg with the Isoprime unit [40], about 120 W h/mg with the Octopus unit [38], but only about 5.5 W h/mg with the Gradiflow BF 200 IET unit. (These comparisons do not take into account the energy costs of cooling and pumping). Thus, the modified Gradiflow BF200 IET unit compares favorably with the other preparative isoelectric focusing and trapping technologies in terms of both production rates and direct electrophoretic power consumption. Both of these advantages are believed to stem from the very short electrophoretic migration distances that exist in the separation cartridge of the modified Gradiflow BF 200 IET apparatus.

The long-term stability of the cathodic isoelectric membrane in contact with the high pH catholyte was not as good as we would have liked. No degradation was observed either on the separation membrane (p*I* = 3.7) or the anodic electrode membrane (p*I* = 3.0).

4. Conclusions

This brief paper has shown that the Gradiflow BF200 IET unit, which was developed for the binary, isoelectric trapping separation of proteins, could be successfully modified and used for the separation of ampholytic enantiomers as well. The production rates and electrophoretic energy consumption values observed with the Gradiflow BF200 IET unit were more favorable than those we obtained with the continuous free-flow electrophoretic device, the Octopus and another IET device, the Isoprime. We believe that it is the combination of the short electrophoretic transfer distance, the higher electric field strength and the large effective surface area of the isoelectric membranes that leads to the rapid separation speeds in the modified Gradipore BF200 IET unit.

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References

[1] Z.S. Horvath, G.L. Corthals, C.W. Wrigley, J. Margolis, Electrophoresis 15 (1994) 968.
[2] J. Margolis, G.L. Corthals, Z.S. Horvath, Electrophoresis 16 (1995) 98.
[3] Z.S. Horvath, A.A. Gooley, C.W. Wrigley, J. Margolis, K.L. Williams, Electrophoresis 17 (1996) 224.
[4] G.L. Corthals, J. Margolis, K.L. Williams, A.A. Gooley, Electrophoresis 17 (1996) 771.
[5] G.L. Corthals, M.P. Molloy, B.R. Herbert, K.L. Williams, A.A. Gooley, Electrophoresis 18 (1997) 317.
[6] G.L. Corthals, B.M. Collins, B.C. Mabbutt, K.L. Williams, A.A. Gooley, J. Chromatogr. A 773 (1997) 299.

[7] S. Lim, H.P. Manusu, A.A. Gooley, K.L. Williams, D.B. Rylatt, J. Chromatogr. A 827 (1998) 329.
[8] D.B. Rylatt, M. Napoli, D. Ogle, A. Gilbert, S. Lim, C.H. Nair, J. Chromatogr. A 865 (1999) 145.
[9] T.M. Thomas, E.E. Shave, I.M. Bate, S.C. Gee, S. Franklin, D.B. Rylatt, J. Chromatogr. A 944 (2001) 161.
[10] A. Gilbert, M. Evtushenko, C.H. Nair, Ann. NY Acad. Sci. 936 (2001) 625.
[11] M. Faupel, B. Barzaghi, C. Gelfi, P.G. Righetti, J. Biochem. Biophys. Methods 15 (1987) 147.
[12] P.G. Righetti, B. Barzaghi, M. Faupel, J. Biochem. Biophys. Methods 15 (1987) 163.
[13] B. Barzaghi, P.G. Righetti, M. Faupel, J. Biochem. Biophys. Methods 15 (1987) 177.
[14] P.G. Righetti, B. Barzaghi, M. Luzzana, G. Manfredi, M. Faupel, J. Biochem. Biophys. Methods 15 (1987) 189.
[15] P. Wenger, M. Dezuanni, P. Javet, C. Gelfi, P.G. Righetti, J. Biochem. Biophys. Methods 15 (1987) 29.
[16] P.G. Righetti, B. Barzaghi, M. Faupel, Trends Biotechnol. 6 (1988) 121.
[17] P.G. Righetti, E. Wenisch, M. Faupel, J. Chromatogr. 475 (1989) 293.
[18] P.G. Righetti, E. Wenisch, A. Jungbauer, H. Katinger, M. Faupel, J. Chromatogr. 500 (1990) 681.
[19] C. Ettori, P.G. Righetti, C. Comninellis, Electrophoresis 13 (1992) 55.
[20] E. Wenisch, P.G. Righetti, W. Weber, Electrophoresis 13 (1992) 668.
[21] C. Ettori, P.G. Righetti, C. Chiesa, F. Frigerio, G. Galli, G. Grandi, J. Biotechnol. 25 (1992) 307.
[22] P.G. Righetti, M. Chiari, E. Wenisch, Analusis 21 (1993) M21.
[23] E. Wenisch, P. Schneider, S.A. Hansen, R. Rezzonico, P.G. Righetti, J. Biochem. Biophys. Methods 27 (1993) 199.
[24] E. Wenisch, K. Vorauer, A. Jungbauer, H. Katinger, P.G. Righetti, Electrophoresis 15 (1994) 647.
[25] M. Chiari, M. Nesi, P. Roncada, P.G. Righetti, Electrophoresis 15 (1994) 953.
[26] W. Weber, E. Wenisch, N. Gunther, U. Marnitz, C. Betzel, P.G. Righetti, J. Chromatogr. A 679 (1994) 181.
[27] A. Bossi, P.G. Righetti, Electrophoresis 16 (1995) 1930.
[28] A. Bossi, P.G. Righetti, C. Visco, U. Breme, M. Mauriello, B. Valsasina, G. Orsini, E. Wenisch, Electrophoresis 17 (1996) 1932.
[29] A. Bossi, P.G. Righetti, E. Riva, L. Zerilli, Electrophoresis 17 (1996) 1234.
[30] P.G. Righetti, A. Bossi, Anal. Biochem. 247 (1997) 1.
[31] P.G. Righetti, A. Bossi, E. Wenisch, G. Orsini, J. Chromatogr. B 699 (1997) 105.
[32] M. Perduca, A. Bossi, L. Goldoni, H.L. Monaco, P.G. Righetti, Electrophoresis 21 (2000) 2316.
[33] B. Herbert, P.G. Righetti, Electrophoresis 21 (2000) 3639.
[34] P.G. Righetti, C. Ettori, P. Chafey, J.P. Wahrmann, Electrophoresis 11 (1990) 1.
[35] P. Glukhovskiy, G. Vigh, Anal. Chem. 71 (1999) 3814.
[36] A.M. Rizzi, L. Kremser, Electrophoresis 20 (1999) 2715.
[37] A.M. Rizzi, L. Kremser, Electrophoresis 20 (1999) 3410.
[38] I. Spanik, P. Lim, G. Vigh, J. Chromatogr. A 960 (2002) 241.
[39] Amersham Pharmacia, IsoPrime Manual, Amersham Pharmacia Biotechnology, San Francisco, CA, USA, 1999.
[40] P. Glukhovskiy, T.A. Landers, G. Vigh, Electrophoresis 21 (2000) 762.
[41] D. Ogle, A. Ho, T. Gibson, D.B. Rylatt, E. Shave, P. Lim, G. Vigh, J. Chromatogr. A 979 (2002) 1.
[42] I. Spanik, G. Vigh, J. Chromatogr. A 979 (2002) 2.