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Preparative-scale isoelectric trapping separations in methanol–water mixtures

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Abstract

The typically low aqueous solubilities of small, hydrophobic organic ampholytic molecules limit the production rates that can be achieved in their isoelectric trapping (IET) separations and call for the use of hydro-organic mixtures as solvents. The compatibility of methanol–water mixtures and poly(ethylene terephthalate) substrate-supported isoelectric polyacrylamide hydrogels, developed for binary IET separations in a Gradiflow BF200IET unit, was investigated. The isoelectric polyacrylamide-based hydrogels retained their functional and mechanical integrities when the methanol concentration in the hydro-organic solvent mixture was kept at or below 25% (v/v). The utility of the hydro-organic media was demonstrated in the purification of a hydrophobic ampholytic compound, technical grade 4-hydroxy-3-(morpholinomethyl) benzoic acid. Production rates as high as 7 mg/h were achieved using small, 15 cm^2 active surface area isoelectric membranes.
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1. Introduction

The isoelectric membrane-based multicompartmental electrolyzer (MCE) [1,2] has been used mostly for the isoelectric trapping (IET) separation of proteins (for an excellent review, see, e.g., [3]). In IET, isoelectric membranes with different isoelectric points (p*I* values) form the separation compartments of the MCE and the proteins of interest are trapped in the respective compartments in their pure form, in isoelectric state, free of background electrolyte components [1,2]. We are aware of only a few papers that describe the preparative-scale IET separation of small organic molecules, such as enantiomers: the first one reports the use of the IsoPrime system [4], the second one the use of the modified BF200IET Gradiflow system [5]. An earlier paper discusses the use of IET for the production of a narrow carrier ampholyte cut, $6.7 < pI < 7.3$, but not the production of a single component [6].

The Gradiflow BF200IET system was designed for binary separation of mixtures of ampholytic components [7]. It has four compartments: an anode compartment, an anodic separation compartment, a cathodic separation compartment and a cathode compartment. The compartments are connected via isoelectric membranes that also act as anti-convective barriers between the compartments. The p*I* values of the isoelectric membranes increase from the anodic membrane, through the separation membrane to the cathodic membrane. The sample mixture flows through shallow (500 μm deep) anodic and cathodic separation compartments, while the electric field applied orthogonally to the direction of the hydraulic flow transports through the isoelectric membranes the ampholytic components whose p*I* value is different from that of the isoelectric membrane. Components whose p*I* value is higher than that of the anodic membrane are prevented from moving into the anode compartment and components whose p*I* value is lower than that of the cathodic membrane are prevented from moving into the cathode compartment. Since the electrophoretic migration distance is very short in the BF200IET unit (500 μm), and even low applied potentials result in high field strengths, the separation speed can be very fast [5].

So far, the isoelectric membranes used in any IET system were synthesized by radical copolymerization of the appropriate Immobiline chemicals, acrylamide and *N*,*N*′-methylenebisacrylamide [8,9], and were operated in aqueous media [3]. Since small organic ampholytic molecules often have very low solubilities in water, even when they are in non-isoelectric state, we were interested to find out if acrylamide-based isoelectric membranes could

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efficiently operate as isoelectric barriers in hydro-organic media, such as methanol–water mixtures, which are often better solvents for small, hydrophobic organic molecules than pure water. The objective of this brief paper is to describe our first experiences with the use of hydro-organic media that contained up to 50% (v/v) methanol in the IET separation of aminocarboxylic acids in a Gradiflow BF200IET system.

2. Experimental

2.1. Chemicals

Electrolyte components glutamic acid (Glu), lysine (Lys), phosphoric acid, sodium hydroxide, methylcellulose (MC, average molecular mass 65000) and HPLC-grade methanol (MeOH), as well as the samples, 4-hydroxy-3-(morpholinomethyl)benzoic acid (HMMB), *m*-aminobenzoic acid (MABA) and 3-pyridinepropionic acid (3PPA) were obtained from Aldrich (Milwaukee, MI, USA), while carrier ampholytes Pharmalyte p*I* 3–10 were from Sigma (St. Louis, MO, USA). All solutions were freshly prepared using deionized water from a Milli-Q unit (Millipore, Milford, MA, USA).

The samples collected in the IET separation were analyzed by full-column icIEF [10] as described in [11]. For icIEF, the anolyte and catholyte were 80 mM phosphoric acid and 100 mM sodium hydroxide, respectively, and both solutions contained 0.1% MC to minimize the electro-osmotic flow. The carrier ampholyte stock solution contained 3.2% carrier ampholytes (p*I* 3–10) and 0.1% MC. This solution was used to dilute the samples collected from the preparative-scale IET separation, at a rate of 4–10 μl sample to 196–190 μl of the carrier ampholyte stock solution, to make a final sample volume of 200 μl.

2.2. Analytical equipment

The fractions collected during the preparative-scale IET separations were analyzed by full-column icIEF using an iCE280 unit (Convergent Biosciences, Toronto, Canada) that was equipped with an Alcott 718AL autoinjector and a 96-well microtiter plate adapter (Alcott, Norcross, GA, USA). The separation cartridge of the iCE280 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 3 min and a focusing time of 4.5 min [11]. The electropherograms were processed by the EZ Chrom software (Scientific Software, Pleasanton, CA, USA).

2.3. Preparative-scale IET equipment

The preparative-scale IET separations were obtained with a BF200IET unit (Gradipore, French's Forest, NSW, Australia) [7]. The block diagram of the system is shown in Fig. 1. The active surface area of each membrane in the separation cartridge was about 15 cm^2, the electrophoretic migration distance about 500 μm, and the separation compartment volume about 0.8 ml.

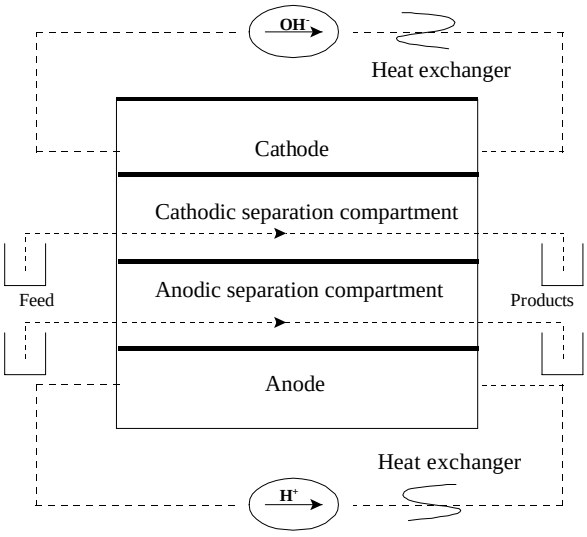

Fig. 1. Schematic of the BF200IET unit.

All isoelectric membranes were polyacrylamide-based (Gradipore) and had a nominal thickness of about 150 μm. The anolyte, catholyte and sample solutions were pumped from jacketed reservoirs that were thermostated by an antifreeze solution flowing through a Model 1106 chiller (VWR, Bristol, CT, USA). The anolyte and catholyte were recirculated at a flow rate of 2 l/min, the sample streams were pumped through the separation compartments in single-pass mode [5], at a flow rate of 30 ml/min. At the end of each pass, 0.5 ml aliquots were taken at the exit ports of all four compartments (anode, anodic separation compartment, cathodic separation compartment, cathode) and analyzed by the iCE280 unit. The separation potential was provided by a 900 V, 1200 mA dc power supply (E-C Apparatus, Holbrook, NY, USA) that could be operated in constant potential or constant current mode.

3. Results and discussion

First, the ability of the polyacrylamide-based isoelectric membranes to function in methanol–water mixtures was tested. Small organic ampholytic components (MABA, pI = 3.9, and 3PPA, pI = 4.8) that have relatively low aqueous solubilities were selected for the IET test in a methanol–water (25:75, v/v) mixture as solvent. The anolyte was 15 mM Glu, the catholyte 15 mM Lys, both dissolved in a methanol–water (25:75, v/v) mixture. The anodic membrane had a p*I* of 3.0, the separation membrane a p*I* of 4.2, the cathodic membrane a p*I* of 7.8. The separation was carried out in constant potential mode at

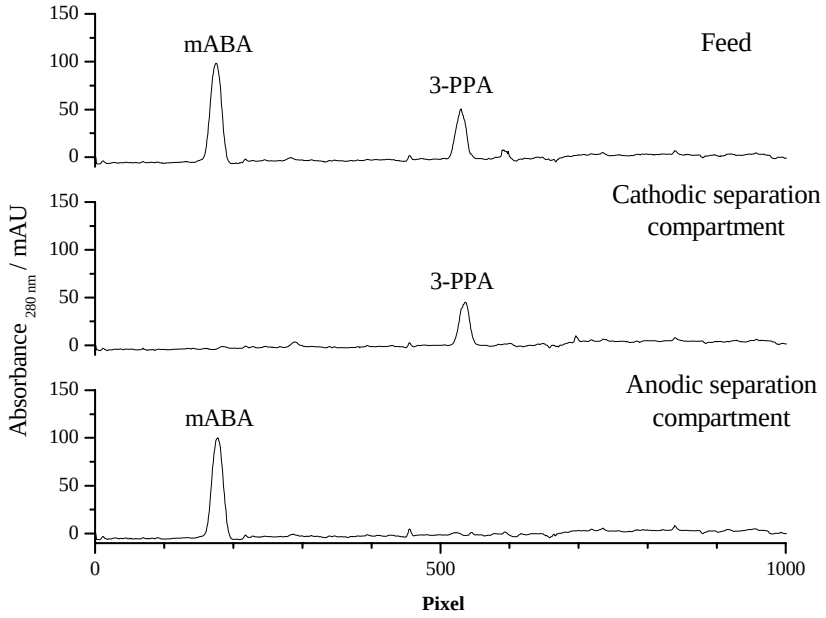

Fig. 2. icIEF separation of the MABA : 3PPA feed sample (top panel), aliquots collected after 30 min at the exit ports of the cathodic separation compartment (middle panel) and the anodic separation compartment (bottom panel) of the Gradiflow BF200IET unit. Nominal pI values of the isoelectric membranes: pI = 3.0 (anodic), pI = 4.2 (separation), pI = 7.8 (cathodic). Separation medium: 25% (v/v) methanol in water, sample feed rate: 30 ml/min.

950 V for 1 h, and created an initial current of 25 mA and final current of 11 mA. The results of the icIEF analysis of the original sample, and the samples collected after 30 min at the exit ports of the cathodic separation compartment and anodic separation compartment are shown in the top, middle and bottom panels of Fig. 2. Clearly, IET separation has been achieved indicating that the polyacrylamide-based isoelectric membranes retain enough of their buffering capacities to function as effective isoelectric barriers in the methanol–water (25:75, v/v) medium for at least 1 h.

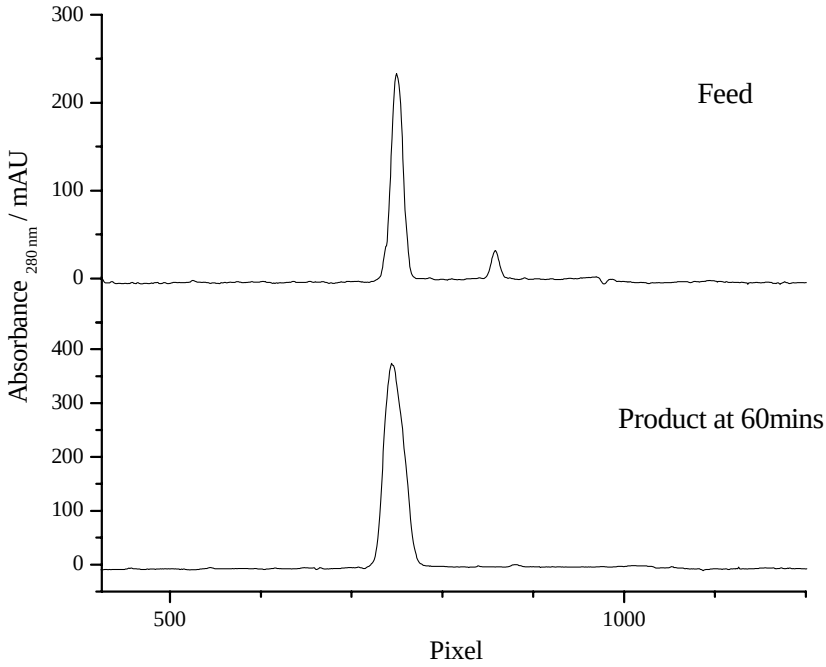

Fig. 3. icIEF separation of the technical grade 4-hydroxy-3-(morpholinomethyl) benzoic acid feed sample (top panel) and purified fraction collected at the exit port of the cathodic separation compartment of the Gradiflow BF200IET unit after 60 min of electrophoresis (bottom panel). Nominal pI values of the isoelectric membranes: pI = 4.2 (anodic), pI = 6.0 (separation), pI = 7.8 (cathodic). Separation medium: 25% (v/v) methanol in water, sample feed rate: 30 ml/min.

Next, the same separation was repeated using a 30% (v/v) methanol–water mixture as solvent in all compartments. The separation was again carried out in constant potential mode at 950 V; the resulting electrophoretic currents were lower due to the lower conductivities caused by the higher methanol concentration. The results of the icIEF analysis show that though the separation was completed in about 45 min, the cathodic membrane began to slowly leak after about 30 min of continuous electrophoresis. Thus, though the membranes still functioned in methanol–water (30:70, v/v) mixtures as isoelectric barriers, the long-term stability of the cathodic membrane was not satisfactory.

In the final stability test, the methanol concentration was increased to 50% (v/v), and the same separation was repeated. All membranes began to leak after about 10 min of electrophoresis. Visual inspection of the membranes after the run indicated that the polyacrylamide hydrogel layer collapsed onto the poly(ethylene terephthalate) substrate.

To demonstrate the suitability of the hydro-organic medium for real-life preparative IET separations, technical grade HMMB (90%, w/w, active component content) that we use as a p*I* marker for the iCE280 system was purified to homogeneity. The hydro-organic medium contained 25% (v/v) methanol. The anolyte was 15 mM Glu, the catholyte 15 mM Lys. The anodic, separation and cathodic membranes had p*I* values of 4.2, 6.0 and 7.8. The initial sample stream contained 2 mM HMMB, 30 ml of it was filled into the solution reservoir of the anodic separation compartment. The initial solution in the cathodic separation compartment was 25% (v/v) methanol in water (30 ml). Complete purification (product purity greater than 99%) required 60 min. The results of the iCE280 analysis of the initial sample (top panel) and the purified target (bottom panel) are shown in Fig. 3. The production rate achieved with the 15 cm^2 membranes is about 7 mg/h.

4. Conclusions

This brief paper has shown that poly(ethylene terephthalate) substrate-supported polyacrylamide-based isoelectric membranes, developed for binary IET protein separations in the Gradiflow BF200IET unit, could be successfully used in hydro-organic media containing up to 25% (v/v) methanol. The solubility of a hydrophobic organic compound, 4-hydroxy-3-(morpholinomethyl) benzoic acid was sufficiently high (2 mM) in this medium to permit its meaningful preparative-scale IET separation with production rates of about 7 mg/h and product purities greater than 99%. Though the presence of methanol probably alteres the p*I* values of the analytes and the isoelectric membranes to a different extent, this is not a major impediment to the use of hydro-organic solvent mixtures, because the appropriate separation membrane can still be selected in a few simple experiments from a series of separation membranes with closely spaced nominal (aqueous) p*I* values (e.g., $\Delta pI = 0.1$).

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