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FOCUSING COUNTERPARTS OF ELECTRICAL FIELD FLOW FRACTIONATION AND CAPILLARY ZONE ELECTROPHORESIS

ELECTRICAL HYPERLAYER FIELD FLOW FRACTIONATION AND CAPILLARY ISOELECTRIC FOCUSING

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SUMMARY

Efforts are currently being undertaken to develop electrical hyperlayer field flow fractionation (EHFFF) and capillary isoelectric focusing (CIEF) as fully instrumental approaches for the analysis of amphoteric samples, such as peptides and proteins. In these techniques a pH gradient parallel to an applied electric force field is employed for sample discrimination according to differences in isoelectric points. EHFFF is performed in a thin ribbon-like channel containing a mobile phase with the electric field perpendicular to the flow direction, whereas CIEF utilizes a quiescent solution with the electric field parallel to the column axis. The principle of EHFFF, the design of EHFFF instrumentation and first results are discussed together with the differences and similarities between EHFFF and CIEF.

INTRODUCTION

Efforts are currently being undertaken in our laboratory to develop electrical hyperlayer field flow fractionation (EHFFF) and capillary isoelectric focusing (CIEF) as fully instrumental approaches for the analysis of amphoteric samples, such as peptides and proteins. EHFFF and CIEF represent the focusing counterparts of electrical field flow fractionation (EFFF) and capillary zone electrophoresis (CZE), respectively[1,2]. CZE[3–7] is conducted in a continuous buffer where the samples are the only discontinuities present. Under the influence of an electric field, sample zones migrate without exhibiting any steady state behavior and, thus, their shape and position continuously change with time (Fig. 1a). In this technique, separation is based upon differences in net mobility. In CIEF[8–12], sample components are sorted according to their isoelectric points in an equilibrium gradient. Typically, a mixture of carrier ampholytes is exposed to an electric field in order to establish a gradient of pH

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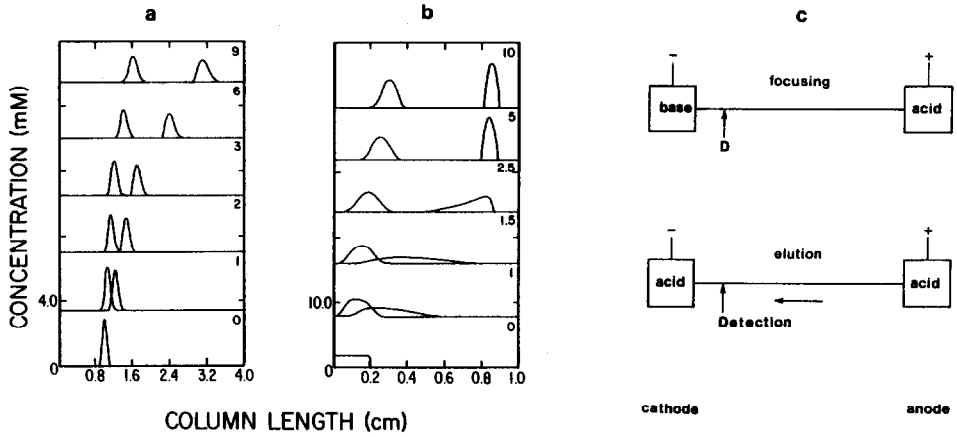

Fig. 1. Schematic representation of the separation dynamics of two sample components in CZE (a) and CIEF (b), together with the two sequential stages in CIEF, focusing and elution (c). D = detector. Computer simulated concentration profiles of two sample components in CZE (a) and CIEF (b) are shown at the time point indicated in the upper righthand corner of each plot. Each successive time point is offset from the previous one by a constant amount for presentation purposes.

increasing from anode to cathode. Proteins and other amphoteric compounds can be separated in this gradient provided their isoelectric points are sufficiently different. Unlike CZE, CIEF sample zones attain a stationary steady state (Fig. 1b). Thus, for detection by an on-column sensor placed toward the column end, the separative pattern must be mobilized [*e.g.* electromobilized (Fig. 1c)] after focusing.

The development of field flow fractionation (FFF) in the last two decades represents an important contribution to the separation of macromolecules and particles[13–15]. FFF is based on the simultaneous influence of an external force field and a flow velocity profile on the constituents undergoing separation. In EFFF, for example, an electric field applied perpendicular to the flow direction acts as the differential retarding force (Fig. 2a)[16–21]. This force causes charged solute molecules to accumulate in layers of distinct thickness near one channel wall. Each solute is then transported by flow along the channel at a rate determined by the mean thickness of its layer. In hyperlayer field flow fractionation (HFFF) separation is based on partitioning by the concomitant presence of an applied force field and an equilibrium gradient[2,22]. In contrast to regular FFF methods, most of the focused solutes in HFFF do not reach the walls of the channel, thereby excluding interactions between the sample components and the column walls. In EHFFF a pH gradient is used in addition to the electric field, the same gradient combination as in CIEF. Amphoteric samples are condensed in layers away from the walls, where the pH value equals their isoelectric point, as is illustrated in Fig. 2b. To date, sedimentation HFFF[2,22–26], in which a density gradient is used in addition to the sedimentation field, and flow HFFF[27], have been discussed. In this paper, the use of an electrically produced pH gradient across the thin dimension of an FFF ribbon-like channel is described. The design of EHFFF instrumentation and first results are discussed together with the differences and similarities between EHFFF and CIEF.

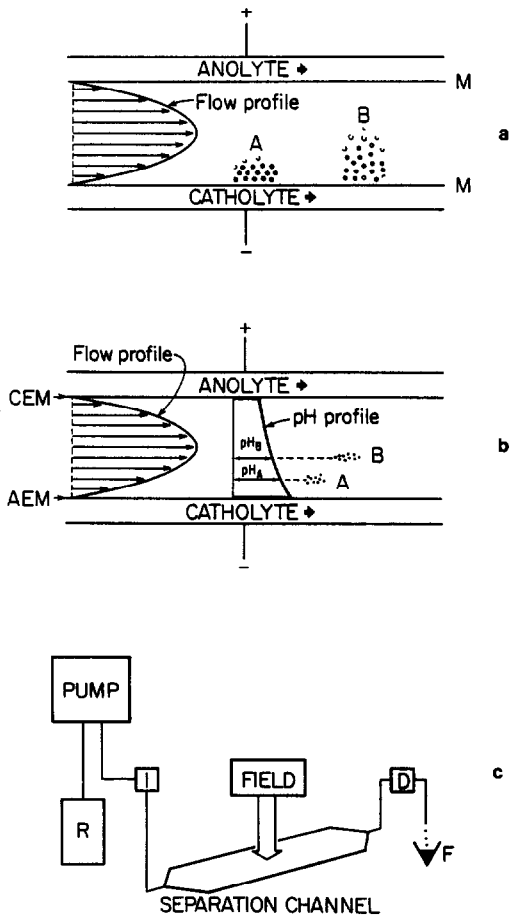

Fig. 2. Schematic representation of (a) EFFF, (b) EHFFF and (c) an FFF apparatus. M = dialysis membrane; CEM = cation-exchange membrane; AEM = anion-exchange membrane; R = buffer reservoir; I = sample injection port; D = detector; F = fraction collector.

EXPERIMENTAL

CIEF experiments were performed with the Tachophor 2127 (LKB, Bromma, Sweden), as described in detail elsewhere[9]. The Tachophor features an air-cooled PTFE capillary of 0.5 mm I.D. and 10 cm length, mounted between the dual detection block (LKB 2127-140) and a laboratory-built electrode compartment. A syringe was employed to insert the mixture of sample and carrier components into the focusing capillary. The cathode was on the detection side (leading electrolyte compartment of Tachophor). After focusing, the cathodic solution was replaced by the anolyte permitting electromobilization of the separative pattern past the detection block. Conductivity and absorbance measurements were continuously made during both the focusing and elution stages. The experiments were run at either constant current or constant voltage, provided by a Spellman RHR 30P30/CR/FG high-voltage d.c. power supply (2 mA max; 30 kV).

A schematic representation of an EHFFF apparatus is depicted in Fig. 2c. The relation to a high-performance liquid chromatography (HPLC) system in which the chromatographic column is replaced by the FFF trough is obvious. The EHFFF separation cell construction was similar to that used for EFFF by Giddings *et al.*[15]. It features a ribbon-like channel (2 × 20 × 0.025 cm) formed by a PTFE spacer which is sandwiched between ion-exchange membranes (Ionics, Watertown, MA, U.S.A.) by use of two Plexiglass blocks (Fig. 3). These blocks contain the electrode reservoirs, as well as fittings for sample, carrier buffer, catholyte and anolyte insertion and drain. A Model 2150 HPLC pump, a Model 2158 Uvicord absorbance monitor with an 8-μl HPLC flow cell, a Model 2195 pH/ion monitor and, for application of anolyte and catholyte, a Model 2132 Microperpex peristaltic pump (all from LKB) were the peripheral instruments (Fig. 2c). Samples were injected with a microliter syringe. A Kepco APH 2000 M power supply was employed for application of the electric force field. The setup was used in the flow-injection mode with and without an applied electric field, as well as in a stopped-flow mode for focusing in quiescent solution, followed by elution of the focused solutes.

The simple buffer mixtures used in this study consisted of three amino acids,

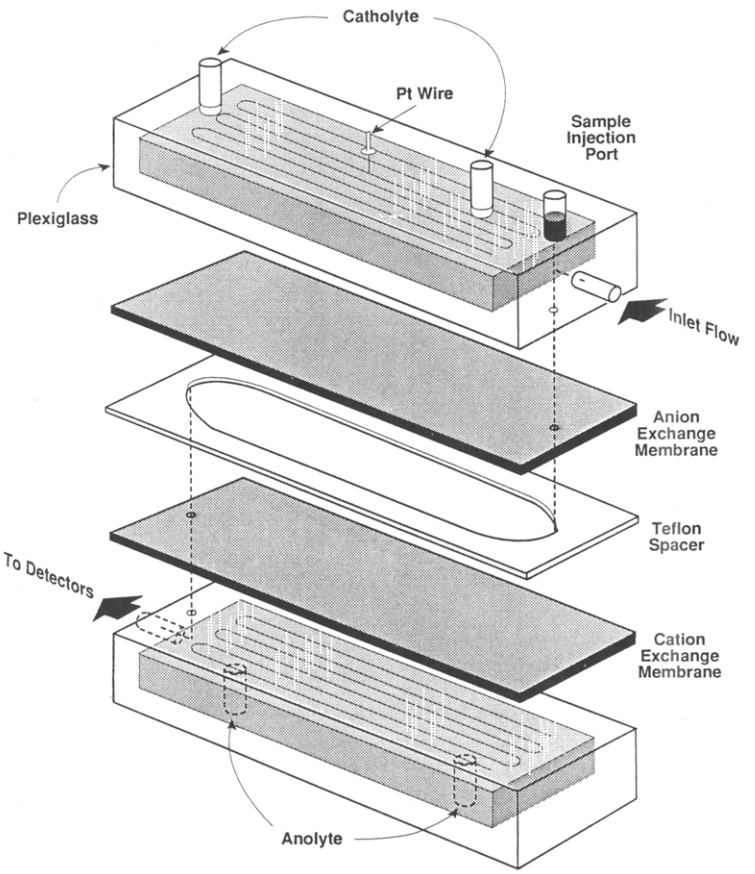

Fig. 3. Exploded assembly of the EHFFF separation cell.

glutamic acid (Fisher Scientific, West Haven, CT, U.S.A.), cycloserine (Sigma, St. Louis, MO, U.S.A.) and arginine (Sigma). The synthetic carrier ampholytes employed were 3.5–10 pH range Ampholine (LKB). Model protein mixtures were prepared from any number of the following: bovine albumin (Alb, Miles Laboratories, Elkhart, IN, U.S.A., pI = 4.9), canine hemoglobin (Hb, isolated locally, pI = 7.3), bovine ribonuclease A (Sigma, pI = 9.6), and equine cytochrome c (Sigma, pI = 10.3). Solutions of phosphoric acid (anolyte) and sodium hydroxide (catholyte), 100 mM each, were employed as electrode buffers for CIEF and EHFFF. Hydroxypropylmethyl cellulose (HPMC) was from Sigma.

RESULTS AND DISCUSSION

The generation of the lateral pH gradient in EHFFF is analogous to the establishment of natural pH gradients in isoelectric focusing. Based on theoretical and practical considerations, the advantages of EHFFF compared to CIEF are manyfold, but the following two are especially important. Firstly, the voltage applied in EHFFF is effectively multiplied by the ratio of column length to width. Because the pH gradient is generated across the smallest channel dimension, much lower applied potentials produce field strengths equivalent to those present in CIEF. Secondly, EHFFF avoids the difficulties of detection in CIEF associated with the production of a stationary steady state. Without the use of scanning or array detectors, the focused proteins in CIEF must be mobilized in order to pass them across a sensor. Fig. 4 shows

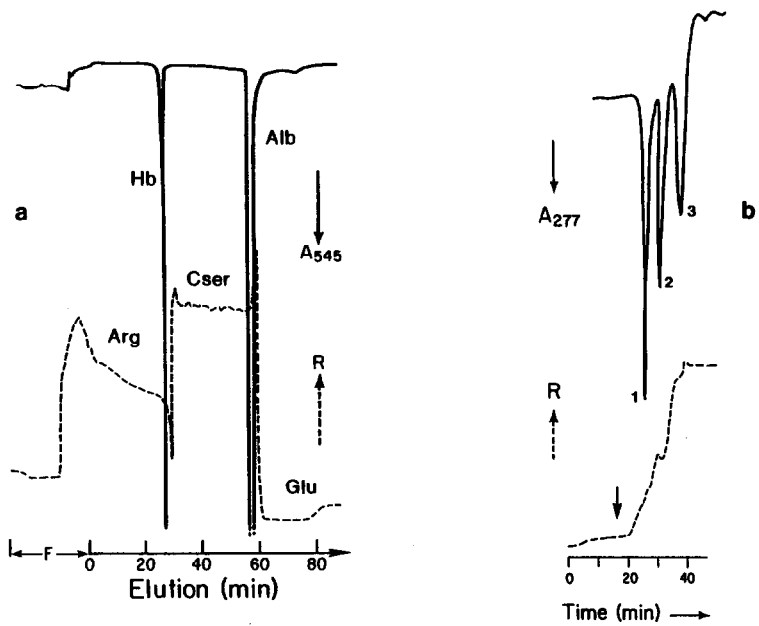

Fig. 4. (a) CIEF conductivity data (expressed as resistance, R) and absorbance data at 545 nm of Hb and Alb focused in a three-component buffer comprising glutamic acid (Glu, 5 mM), cycloserine (Cser, 5 mM) and arginine (Arg, 30 mM) and (b) corresponding CIEF conductivity (R) and absorbance (277 nm) data of cytochrome c (0.18 mg/ml; 1), ribonuclease A (0.36 mg/ml; 2) and Hb (0.08 mg/ml; 3) in 1% Ampholine, 8 mM arginine and 1% HPMC. Focusing occurred (a) within 20 min at 3 kV and (b) within 17 min at 2 kV. The arrow in the latter pherogram marks the beginning of elution.

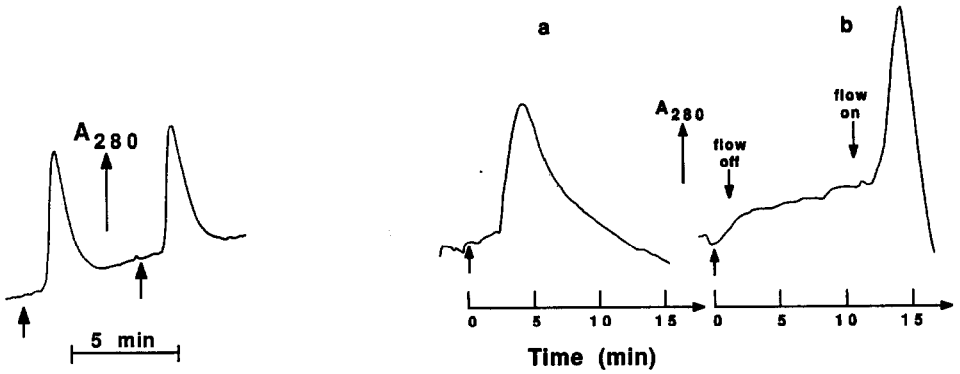

Fig. 5. Flow-injection peaks of 1 μl of 50 mg/ml albumin in a three-component buffer composed of glutamic acid, cycloserine and arginine (10 m*M* each) obtained with the EHFFF apparatus without applied electric field. The data of two consecutive experiments is shown to illustrate reproducibility. The flow-rate was 1.0 ml/min. The arrows mark the time of sample injection. The channel dead volume was about 1 ml.

Fig. 6. Effect of focusing on peak shape in the stopped-flow EHFFF mode. The flow-rate was 0.1 ml/min. The sample and the buffer were the same as for Fig. 5. The flow-injection peak without any electric field is shown in Fig. 6a, and the eluted peak after a focusing period of 10 min without buffer flow is presented in Fig. 6b.

pherograms obtained for the CIEF separation of two model systems of proteins. For detection, the zone structures were electromobilized by replacement of the base (100 m*M* sodium hydroxide) in the cathodic electrode compartment with acid (100 m*M* phosphoric acid) (Fig. 1c) and application of a constant current of 100 μA. This mobilization step significantly prolongs the analysis and might sacrify resolution. EHFFF overcomes this problem by its inherent nature as an elution technique.

The EHFFF apparatus was first tested in the flow-injection mode without application of an electric field. Typical UV responses obtained by injecting 1-μl aliquots of 50 mg/ml albumin into a carrier buffer composed of glutamic acid, cycloserine and arginine (10 m*M* each) are shown in Fig. 5. The buffer flow-rate was 1 ml/min. A ten-fold reduction in the flow-rate produced a broader and more asymmetric peak (Fig. 6a). The asymmetry present in both Figs. 5 and 6a indicates the presence of solute interaction with the channel walls (membranes). Application of the electric field (2.8 V, 2 mA) did not significantly change the elution times of the sample front, the peak maximum and the peak shape. This was true for a flow-rate range of 0.1–0.3 ml/min. Neither could any evidence of separation of proteins be observed. Both results suggested that the length of the separation channel was insufficient.

One way to lengthen the separation channel effectively is to employ a stopped-flow mode of operation. In this mode the sample was first injected without buffer flow, then swept into the beginning of the channel. The electric field was then applied for a specified time without any buffer flow. This allowed focusing to occur before elution was started. The effect on the eluted sample shape, for a focusing time of 10 min at a current of 2 mA (about 2.72 V) followed by an elution in the continued presence of the field at a flow-rate of 0.1 ml/min, is shown in Fig. 6b. The protein sample was found to be eluted as a much sharper zone, indicating a substantial reduction or even exclusion of interactions between the macromolecules and the walls. To our

knowledge, this contribution constitutes the first experimental demonstration of the impact of a lateral pH gradient on the EFFF sample zone. Moreover, these results suggest that EHFFF has the potential of overcoming many of the difficulties encountered in protein analysis by electrical and other FFF techniques, in CZE and in CIEF. Investigations directed toward the exploration of EHFFF as a separation methodology for amphoteric compounds and toward the development of improved separation channels, featuring various types of membranes and non-gassing electrode assemblies, are now underway in our laboratory.

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