POLY(VINYL ALCOHOL)-BASED BUFFERING MEMBRANES FOR ISOELECTRIC TRAPPING SEPARATIONS

A Dissertation

by

HELEN C. FLEISHER CRAVER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Chemistry

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Approved by:

Chair of Committee,	Gyula Vigh
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ABSTRACT

Poly(vinyl alcohol)-based Buffering Membranes for Isoelectric Trapping Separations.

(May 2007)

Helen C. Fleisher Craver, B.S., Saint Louis University

Chair of Advisory Committee: Dr. Gyula Vigh

Isoelectric trapping (IET) in multicompartment electrolyzers (MCE) has been widely used for the electrophoretic separation of ampholytic compounds such as proteins. In IET, the separation occurs in the buffering membranes that form a step-wise pH gradient in the MCE. Typically, buffering membranes have been made by copolymerizing acrylamide with Immobiline compounds, which are acidic and basic acylamido buffers. One major problem, however, is that these buffering membranes are not stable when exposed to high concentrations of acid and base due to hydrolysis of the amide bonds. Poly(vinyl alcohol)-based, or PVA-based, membranes were made as an alternative to the polyacrylamide-based membranes since they provide more hydrolytic and mechanical stability.

Four mid-pH, PVA-based buffering membranes that contain single ampholytes were synthesized. These buffering membranes were used to trap small molecular weight pI markers for up to three hours, and were also used in desalting experiments to remove strong electrolytes from a solution of ampholytes. Additionally, the membranes were used in IET experiments to separate mixtures of pI markers, and to fractionate the major

proteins in chicken egg white. The membranes did not show any degradation when stored in 3 M NaOH for up to 6 months and were shown to tolerate current densities as high as 16 mA/cm^2.

In addition, six series of PVA-based membranes, whose pH values can be tuned over the $3 < pH < 10$ range, were synthesized by covalently binding aminodicarboxylic acids, and monoamines or diamines to the PVA matrix. These tunable buffering membranes were used in trapping experiments to trap ampholytes for up to three hours, and in desalting experiments to remove strong electrolytes from a solution of ampholytes. These tunable buffering membranes were also used in IET experiments to separate proteins, some with pI values that differ by only 0.1 pH unit. The tunable buffering membranes did not show any signs of degradation when exposed to 3 M NaOH for up to 3 months, and could be used in IET experiments with current densities as high as 20 mA/cm^2. These tunable buffering membranes are expected to broaden the application areas of isoelectric trapping separations.

To my husband, Ted, for his love, support, encouragement and patience

To the Fleishers and McCanns for being wonderful and caring parents and siblings, and for helping me become the person that I am today

To the Cravers for welcoming me into their loving family

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NOMENCLATURE

ASC	Anodic separation compartment
ASP	Aspartic acid
ASP-PROP-PVA	PVA-based membrane made with ASP as the buffer, PROP as the titrant, PVA and GDGE
ASP-PVA	PVA-based membrane made with aspartic acid, PVA and GDGE
BGE	Background electrolyte
$BzTMA^+BS^-$	Benzyltrimethylammonium benzenesulfonate
$BzTMA^+PTS^-$	Benzyltrimethylammonium para-toluenesulfonate
CA	Carrier ampholyte
CAR	Carnosine
CDQ-PVA	PVA-based membrane made with a quaternary ammonium group, β-cyclodextrin, PVA and GDGE
CE	Capillary electrophoresis
CSC	Cathodic separation compartment
DACA	Diaminocarboxylic acid
DACA-PVA	PVA-based membrane made with a diaminocarboxylic acid, PVA and GDGE
DAPA	Diaminopropionic acid
DAPA-PVA	PVA-based membrane made with diaminopropionic acid, PVA and GDGE

DEAPA	3-(Diethylamino)propylamine
DEAPA-ASP-PVA	PVA-based membrane made with DEAPA as the buffer, ASP as the titrant, PVA and GDGE
EOF	Electroosmotic flow
GDGE	Glycerol diglycidyl ether
GLU	Glutamic acid
GLU-PVA	PVA-based membrane made with glutamic acid, PVA and GDGE
HIS	Histidine
HIS-PVA	PVA-based membrane made with histidine, PVA and GDGE
HMMB	4-Hydroxy-3-(morpholinomethyl)-benzoic acid
IDA	Iminodiacetic acid
IDA-PVA	PVA-based membrane made with iminodiacetic acid, PVA and GDGE
IEF	Isoelectric focusing
IET	Isoelectric trapping
IPG	Immobilized pH gradient
LAB	Labetalol
LYS	Lysine
LYS-PVA	PVA-based membrane made with lysine, PVA and GDGE
MABA	*Meta*-aminobenzoic acid

MAL	Aminomalonic acid
MAL-PROP-PVA	PVA-based membrane made with MAL acid as the buffer, PROP as the titrant, PVA and GDGE
MCE	Multicompartment electrolyzer
MORPH	4-(2-Aminoethyl)morpholine
MORPH-ASP-PVA	PVA-based membrane made with MORPH as the buffer, ASP as the titrant, PVA and GDGE
MSWIFT	Membrane-separated wells for isoelectric focusing and trapping
ORN	Ornithine
ORN-PVA	PVA-based membrane made with ornithine, PVA and GDGE
PROP	3-Amino-1-propanol
PROP-ASP-PVA	PVA-based membrane made with PROP as the buffer, ASP as the titrant, PVA and GDGE
PVA	Poly(vinyl alcohol)
PYR	1,3,4,6,7,8-Hexahydro-2*H*-pyrimido[1,2-*a*]pyrimidine
SD	Standard deviation
TRIS	Tris(hydroxymethyl)aminomethane
TRIS-ASP-PVA	PVA-based membrane made with TRIS as the buffer, ASP as the titrant, PVA and GDGE

QCD-PVA	PVA-based membrane made with a quaternary ammonium derivative of β-cyclodextrin, PVA and GDGE
Q-PVA	PVA-based membrane made with a quaternary ammonium Group, PVA and GDGE

1. INTRODUCTION

1.1 Isoelectric focusing

1.1.1 Fundamental principles

Isoelectric focusing (IEF) is a technique commonly used to separate ampholytic compounds, especially proteins [1]. In IEF, compounds are separated according to their isoelectric point (pI), which is the pH at which a compound has no net mobility in an electric field. Ampholytic compounds will focus into a region of the pH gradient where the pH is equal to their pI. A pH gradient can be formed using carrier ampholytes (CAs), which are mixtures of low molecular weight aliphatic polyamino-polycarboxylic acids with discrete pI values [1, 2]. CAs have good conductivity and high buffering capacity around their pIs and ensure adequate current and a stable pH gradient during the course of the separation. Various synthetic routes can be used to derive CAs [1, 3, 4]. One major problem encountered in CA-based IEF, however, is that the pH gradient formed is temporally unstable due to anodic and cathodic drift [5].

1.1.2 Immobilized pH gradients

To address the problem of anodic and cathodic drift, IEF in immobilized pH gradients (IPG) was developed [6, 7]. In an IPG, the pH gradient is formed by copolymerizing acrylamide, *N,N'*-methylenebis(acrylamide) and Immobilines (acidic and basic acrylamido buffers [7]).

This dissertation follows the style and format of *Electrophoresis*.

Tables 1 and 2 show the names, structures, and pK_a values of the available Immobilines. The selection of the acrylamido buffer system and the concentration ratio of the acidic and basic components determine the pH inside the gel according to the Henderson-Hasselbach equation (Equation 1):

$$pH = pK_a + \log ([A^-]/[HA]) \quad \text{Eq. 1}$$

Because of the range of Immobilines available, the gel pH can be tuned continuously in either a wide or narrow range. The resolving power of IPGs (expressed as the smallest Δ pI that can be distinguished) is about Δ pI = 0.001. IPG-IEF has many applications [8-12], including first dimension separation of proteins in 2-dimensional gel electrophoresis [13, 14].

1.2 Isoelectric trapping principles

1.2.1 Classical isoelectric trapping

Isoelectric trapping (IET) is based on the principles of IEF, where isoelectric compounds are separated in a pH gradient based on their isoelectric points. In IET, however, there is not a continuous pH gradient formed by carrier ampholytes, but rather a step-wise gradient established by buffering (also known as isoelectric) membranes, with the pH increasing from anode to cathode [15, 16]. Ampholytic sample components are collected in their pure, isoelectric form in chambers of a multicompartment electrolyzer (MCE) [17, 18]. A protein or ampholytic compound in a charged state will migrate to the oppositely charged electrode until it reaches a compartment whose membrane pH values bracket the pI of the compound. The compound will therefore be continuously titrated by the buffering membranes surrounding it, and will remain “trapped” in the

Table 1. Acidic Immobilines, their name, structure, and corresponding pK_a values.

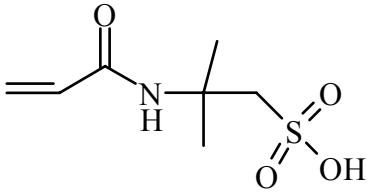
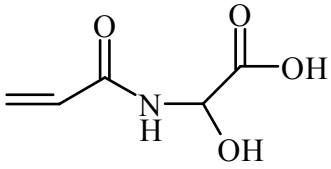
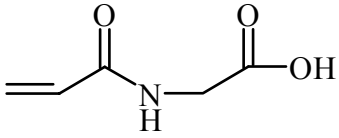
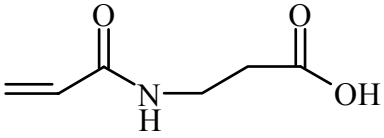
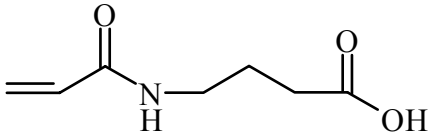
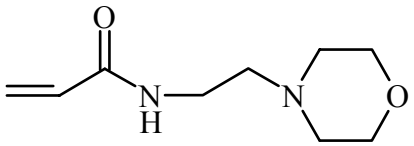
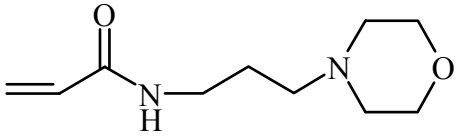
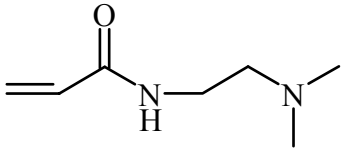
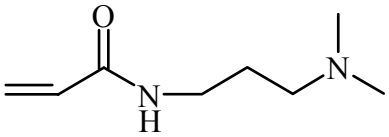
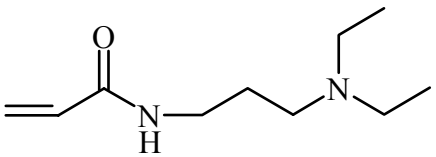
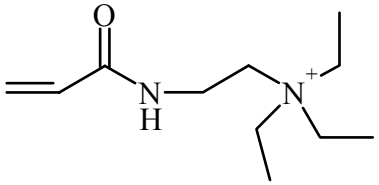
Name	Structure	pK_a
2-acrylamido-2-methylpropane sulfonic acid		1.2
2-acrylamido glycolic acid		3.1
N-acryloyl glycine		3.6
3-acrylamido propanoic acid		4.4
4-acrylamido butyric acid		4.6

Table 2. Basic Immobilines, their name, structure, and corresponding pK_a values.

Name	Structure	pK_a
2-morpholino ethylacrylamide		6.2
3-morpholino propylacrylamide		7
N,N-dimethyl aminoethyl acrylamide		8.5
N,N-dimethyl aminopropyl acrylamide		9.3
N,N-diethyl aminopropyl acrylamide		10.3
QAE-acrylamide		>12

compartment. Strong electrolytes will not be titrated by the buffering membranes and will consequently continue to migrate until they reach the anode or cathode compartment. The buffering membranes will have pore sizes large enough to allow proteins to pass through, yet still prevent convective mixing of the solutions in adjacent compartments allowing for the isolation of proteins or compounds with pIs in a chosen pH range. Figure 1 shows a schematic representation of an IET separation of two proteins, one with a pI of 4 and one with a pI of 8. In this set-up, there are 4 compartments: two separation compartments that contain the protein solutions, and one that contains the anolyte, another the catholyte. The compartments are separated by buffering membranes that buffer at pH 2, 7, and 12. Figure 1a shows the starting conditions of the experiment. In Figure 1b, the potential is applied, and the proteins begin to migrate in the directions indicated by the arrows. After the separation is complete (Figure 1c), the protein with pI 4 will be in the compartment bracketed by the pH 2 and 7 membranes, and the protein with pI 8 will be in the compartment bracketed by the pH 7 and 12 membranes. IET separations have been used for many applications, including the separation of small ampholytic molecules and proteins [16-27], enantiomer separations [28, 29], desalting [30-32], and prefractionation of proteomic samples [24, 33, 34].

1.2.2 pH-biased IET

In classical IET, the separation occurs because of the titration effect in the buffering membranes. When isoelectric compounds are trapped in a sample compartment, they

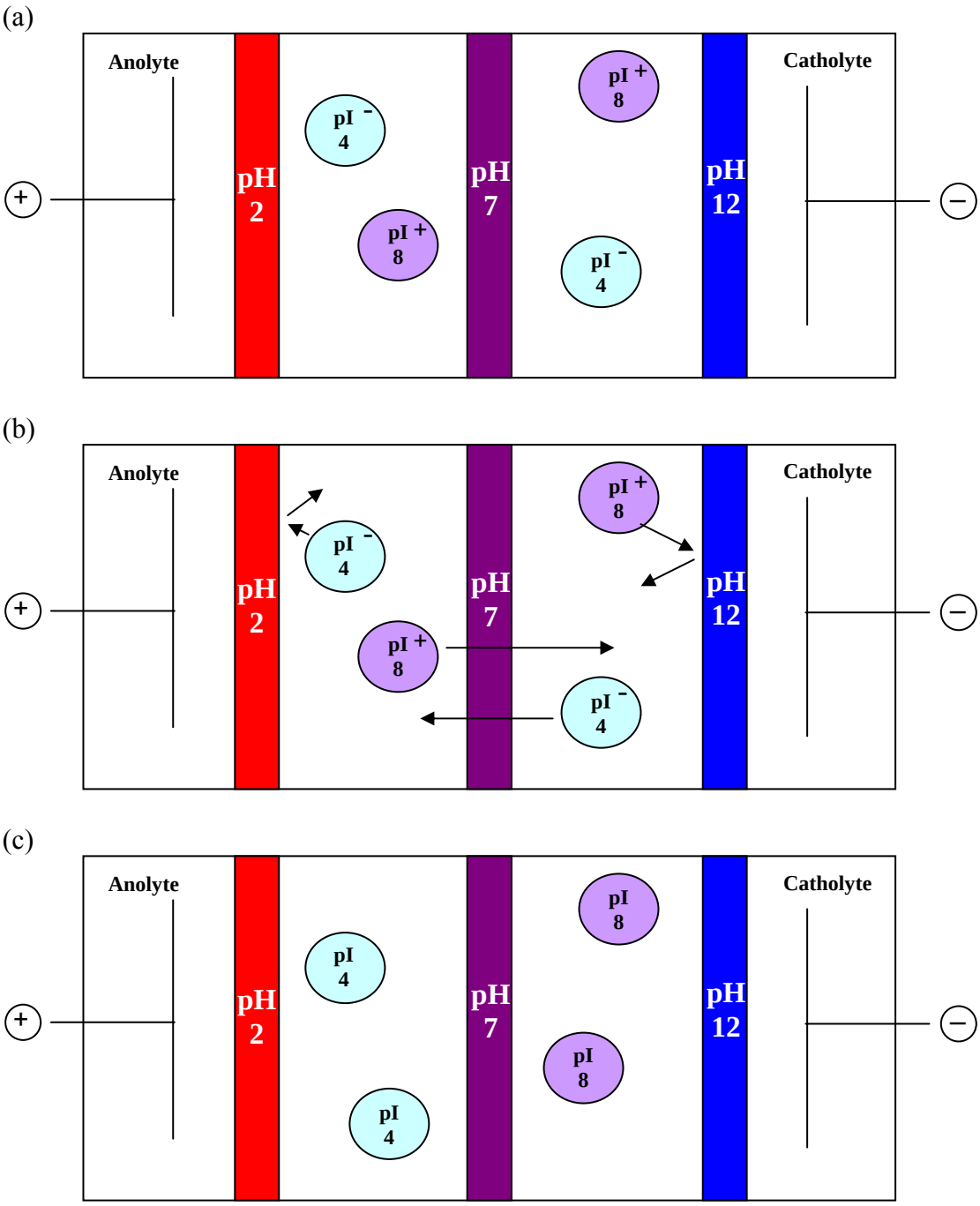


Figure 1. A schematic diagram showing an IET process: (a) Initial set-up of a separation of a pI 4 protein and a pI 8 protein. (b) Potential is applied and proteins migrate toward the oppositely charged electrode until they are titrated by a buffering membrane. (c) Final conditions of separation, with proteins trapped in the compartments that have membranes that bracket their pI values.

are in their pure isoelectric state (or very close to isoelectric state), and are therefore minimally or not at all charged. This leads to poor solubility, slow migration and therefore slow separation of compounds, especially ones whose isoelectric points are very close to the pH of the buffering membrane. Some methods have been utilized to try to improve protein solubility in IET separations, including the addition of organic solvents [35], and the addition of carbohydrate solubilizers [23]. These methods, however, only mitigate the issue of poor solubility and still do not address the problem of long separation times. Establishing a pH even 1 unit away from the pI of a protein can significantly increase the number of charges on the protein, which will increase its electrophoretic mobility, in addition to increasing its solubility [20].

In pH-biased IET, a highly soluble isoelectric buffer whose pI is in between the pH of the surrounding membranes is added to each compartment to maintain a stable pH that is different from the pI of the compound of interest. The target proteins are therefore kept in a non-isoelectric state, which improves their solubility and also increases electrophoretic mobility. These isoelectric buffers are also known as pH-biasers. Good pH-biasers will also be good carrier ampholytes [1] and will therefore have good buffering capacity and a high conductance in isoelectric state. They will also ideally be UV-transparent to facilitate analysis. Several amino acids can be used as pH-biasers, including aspartic acid (pI = 2.7) and glutamic acid (pI = 3.2) [1]. Recently, several new series of isoelectric buffers have been synthesized and used in pH biased IET separations, including quaternary ammonium dicarboxylic acid derivatives [36], and

diamino sulfate derivatives [37]. Shave et al. have used pH-biased IET for the binary separation of proteins in chicken egg white, which resulted in a three-fold increase in production rate and a 30-50% reduction in the specific energy consumption as compared to conventional IET [20].

1.3 Isoelectric trapping devices

1.3.1 Small-scale multicompartment electrolyzers

There are several MCE devices that have been developed for IET, some for the purpose of prefractionation in proteomics analyses [24, 33, 34]. The IsoelectriQ^2, developed by Proteome Systems (Sydney, Australia), is an MCE that can hold up to seven polycarbonate separation compartments, with an intermembrane distance of 2.2 cm and a compartment volume of 5 mL [38, 39]. The device is placed on a cooled platform, which also acts as a magnetic stir plate to stir solutions in the compartment when magnetic stir bars are placed inside. Another small-scale MCE, developed by Zuo and Speicher, is commercially available from Invitrogen (Carlsbad, CA, USA) as the ZOOM^{TM} [40, 41]. This device can hold up to seven Teflon separation compartments, with intermembrane distances of 1.4 cm, and compartment volume of approximately 700 μL. Both the IsoelectriQ^2 and the ZOOM^{TM} need to be operated at below 3 Watts of power since Joule heat removal is inefficient, despite to cooling mechanism with the IsoelectriQ^2.

A recently developed device by Lim and coworkers mitigates the current problem with the present small-scale devices. The device is called membrane-separated wells for isoelectric focusing and trapping (MSWIFT). It facilitates fast and efficient heat dissipation by using 99.8 % non-porous ceramics grade alumina blocks as the sample compartments. It also minimizes the heat conduction path by reducing the distance from the center of the compartment to the wall. In addition, it can provide higher electric field strengths during electrophoresis because of a reduced anode to cathode distance. The MSWIFT has been used for several applications, including desalting experiments, separations of small ampholytic compounds, and separation of proteins in chicken egg white [42].

1.3.2 Preparative-scale, recirculating multicompartment electrolyzers

The recirculation of sample liquid in a MCE offers several benefits. Firstly, it can effectively remove Joule heat by continuously cooling the liquid as it recirculates through the chambers. Also, larger samples volumes can be accommodated, allowing for preparative scale separations. One such device for recirculating IET, and one of the original commercially available MCEs, called the Isoprime, was developed in 1989 by Righetti and coworkers [17, 18]. This device consists of up to six separation compartments that contain 5 mL of solution, with inlets and outlets for sample recirculation, where the flow is orthogonal to the electric field. The membrane to membrane distances are 10 mm, and when all six sample chambers are assembled along with the electrode chambers, the anode to cathode distance is 10 cm. The first results

using the Isoprime showed that the device can efficiently purify high loads of human monoclonal antibody isoproteins with very high resolving power [17, 18]. Later, a large-scale device known as the Rotofor was modified by Shang et al. to be used for IET separations [43]. They demonstrated the trapping of myoglobin and the separation of yeast proteins.

A modified Gradiflow BF200IET has also been developed as an IET device [19]. It performs a binary separation of proteins or isoelectric compounds that are recirculated in one of two sample chambers, where the sample flow is orthogonal to the electric field. One of the major advantages of the Gradiflow technology is that the membrane to membrane distance is only about 1mm, and the anode to cathode distance is only about 8 mm, as shown in Figure 2. These values are much smaller than in other MCEs. This allows for shorter migration distance and higher field strengths, which decrease separation time. The active membrane surface area is 15 cm^2. The modified Gradiflow BF200IET has been used for multiple applications, including separation of chicken egg white proteins [19, 20, 44], depletion of the abundant protein albumin from human plasma [45], and desalting experiments [30-32, 46].

1.4 Buffering membranes

Since IET relies on buffering membranes to effect the separation, there is a need for reliable membranes that buffer at a desired pH value. These buffering membranes need to be stable over the course of the run. They must also be isoelectric and therefore have

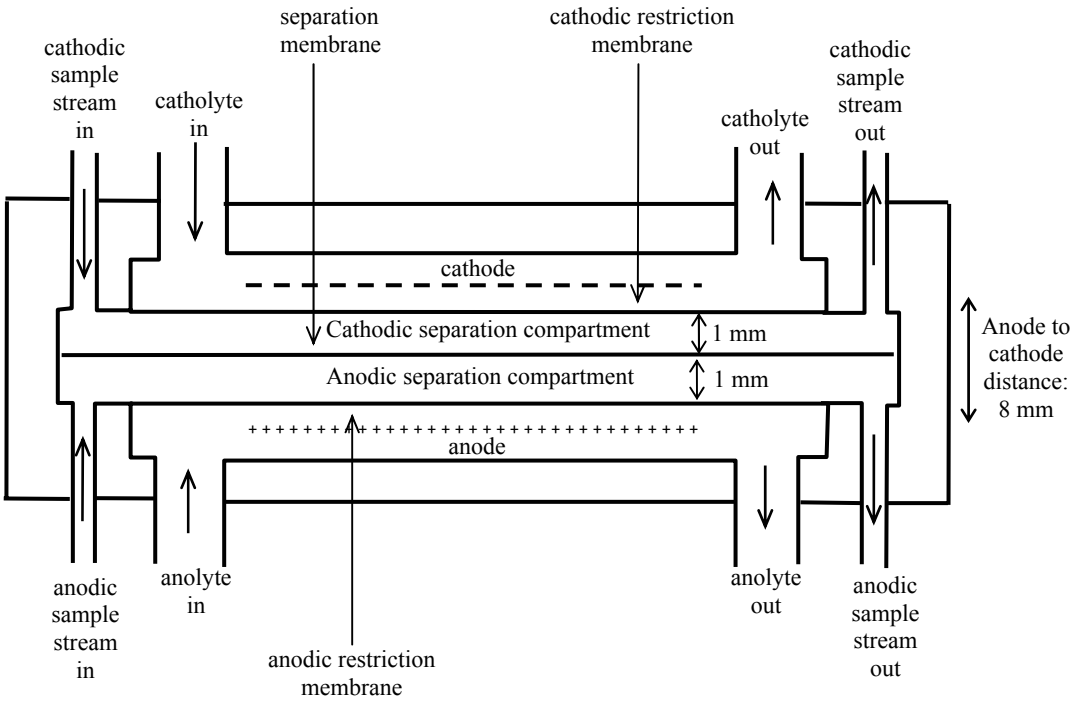


Figure 2. Cross-sectional view of the Gradiflow BF200IET separation cartridge.

a net charge of zero at their isoelectric point. Several synthetic routes have been used to develop buffering membranes [6, 15, 16, 30-32, 47, 48].

1.4.1 Agarose-based buffering membranes

Martin et al. have developed methods for chemically treating agarose to form membranes with attached acidic and basic groups. Agarose forms stable hydrogels, and when cross-linked can withstand the chemical reaction conditions necessary to attach acidic and basic functionalities. These membranes buffer well at their isoelectric point. They were synthesized by soaking filter paper in a 2-8% w/v basic solution of agarose, and cross-linked using epichlorohydrin dissolved in xylene. Acidic functionalities were added by treating the membrane with a solution of sodium chloroacetate in sodium hydroxide. Basic functionalities were introduced by treating the membranes with epichlorohydrin and varying amounts of 0.08-0.12 M diethanolamine in basic solution. The membranes had pH values between 4.8 to 5.5 depending on the concentration of diethanolamine [15, 47]. In a subsequent experiment, buffering membranes were also synthesized by reacting the agarose membrane with Ampholine carrier ampholytes [3] and epichlorohydrin to produce membranes with pH values between 4 and above 10 [15, 47]. One major disadvantage of these membranes is that they do not adequately prevent electroosmotic flow (EOF) when used in IET experiments.

1.4.2 Polyacrylamide-based buffering membranes

Faupel et al. have made polyacrylamide based buffering membranes using the same technology as for IPGs [16]. An acrylamide solution was cross-linked with *N,N'*-methylenebis(acrylamide) and Immobilines (Tables 1 and 2). By adjusting the ratio of the different Immobilines according to the Henderson Hasselbach equation, IPG segments in the low range (pH 3.5-7.2) and high range (pH 7.4-10) were developed. Faupel et al. separated compounds in a segmented IPG apparatus, similar to those used for IET separations. They performed an experiment that removed charged dyes from the central flow-chamber in a desalting experiment. They also separated human adult hemoglobin from a red blood cell lysate from individuals heterozygous for hemoglobin C.

Wenger et al. have also developed buffering membranes with single, discrete and predictable pH values using polyacrylamide-Immobiline based gels cast onto non-woven polypropylene cloth. They determined the pI of the membrane by surrounding the membrane with buffer of known pH. The electroosmotic flow (EOF) across the membrane was then measured. The buffer pH was varied, and the pI of the membrane was determined to be at the pH of the buffer where the EOF across the membrane was zero. At this pH, the number of positive charges will equal the number of negative charges, hence the EOF will be zero, and the isoelectric point will be equal to the pH of the buffer [48]. Polyacrylamide-based buffering membranes have also been used in a

wide variety of applications for small molecule separations, protein separations, and enantiomer separations [16, 24, 48].

1.4.3 Poly(acryloylaminoethoxyethanol)-based buffering membranes

Due to the tendency of polyacrylamide membranes to hydrolyze when exposed to acidic and basic solutions, new schemes have been developed for the synthesis of buffering membranes. Chiari et al. have synthesized membranes using the monomer *N*-acryloylaminoethoxyethanol [49] instead of the acrylamide monomer. They reported that the poly(acryloylaminoethoxyethanol)-based membranes were 500 times more stable than polyacrylamide based membranes, and were also more hydrophilic and had larger porosity due to the higher molecular weight of the monomers. These membranes did not hydrolyze when exposed to, for up to 5 min, 100 mM NaOH solutions, however, when the membranes were exposed to 100 mM NaOH for 10 min, they showed a small percent of hydrolysis, which seemed to make the membranes more acidic by 0.2 pH units [50].

1.4.4 Buffering gel beads

Another disadvantage of polyacrylamide based membranes is that the polyacrylamide matrix must physically adhere to a mechanical support in order to prevent collapsing in IET separations. The support must be rigid but also porous enough to allow the passage of proteins. Polyacrylamide gels do not adhere well to the current supports, which makes the scale-up of membranes for IET separations difficult. Chiari et al. developed a

novel system for IET separations by using a bed of gel beads instead of traditional membranes which consist of gels cast onto supports. The acrylamide buffering beads were synthesized using inverse emulsion polymerization [51]. An emulsion was created by mixing acrylamide, bisacrylamide, Immobilines, and ammonium persulfate, and then N,N,N',N'-tetramethylethylenediamine was added to start the polymerization. The isoelectric beads were successfully used in a focusing experiment of cytochrome c, and also in the IET separation of β-lactoglobulin A and B, carbonic anhydrase, and cytochrome c [52].

1.4.5 Poly(vinyl alcohol)-based buffering membranes

A more recent development in membrane chemistry by our research group that addresses the hydrolysis and support problems of polyacrylamide based membranes is the synthesis of poly(vinyl alcohol), (PVA), based buffering membranes [30-32]. PVA is synthesized by free radical polymerization of vinyl acetate, followed by hydrolysis of poly(vinyl acetate) to form poly(vinyl alcohol). PVA forms hydrogels once cross-linked, and can also be functionalized with acidic and basic functionalities, or ampholytic species by reaction with the hydroxyl groups. It is much more hydrolytically stable than polyacrylamide based hydrogels, and can withstand exposure to acidic and basic solutions over 1 M in concentration [30, 31].

Low pH membranes have been synthesized by dissolving a 20% w/v solution of PVA in 3 M aqueous NaOH, and adding an ampholyte with a well defined pI value. Glycerol

diglycidyl ether (GDGE) is a bifunctional epoxide, and was used to crosslink the PVA strands together to form the hydrogel, and also to act as a tether for the amine-containing ampholytes. Nucleophiles such as amines and hydroxyl groups will react with epoxides, under basic conditions, in an SN2 type of reaction, with the nucleophile attacking the less substituted carbon on the epoxide ring. After reaction with GDGE, the solution was then cast onto a PVA paper support which could then further react with the crosslinker to covalently bind the hydrogel to the support. Since the support is chemically equivalent to the matrix, it does not affect the buffering pH of the membrane. Iminodiacetic acid (IDA) was used to make IDA-PVA membranes, aspartic acid (ASP) was used to make ASP-PVA membranes, and glutamic acid (GLU) was used to make GLU-PVA membranes [31]. Table 3 lists the PVA-based membranes that have been synthesized, the compound incorporated into the matrix to establish the pH of the membrane, and also the range in which the pH value lies for that membrane. Figure 3 represents a possible schematic structure of an IDA-PVA hydrogel, as an example.

One major advantage of incorporating ampholytic compounds into the PVA hydrogel is that if the concentration of the ampholyte changes slightly, it will not affect the overall pH of the membrane, provided that the ampholyte concentration is still at or higher than its “isoelectric concentration”. These membranes have been used in several IET experiments for the separation of small ampholytic molecules and proteins, and have been shown to be a good alternative to polyacrylamide based membranes because of their hydrolytic stability.

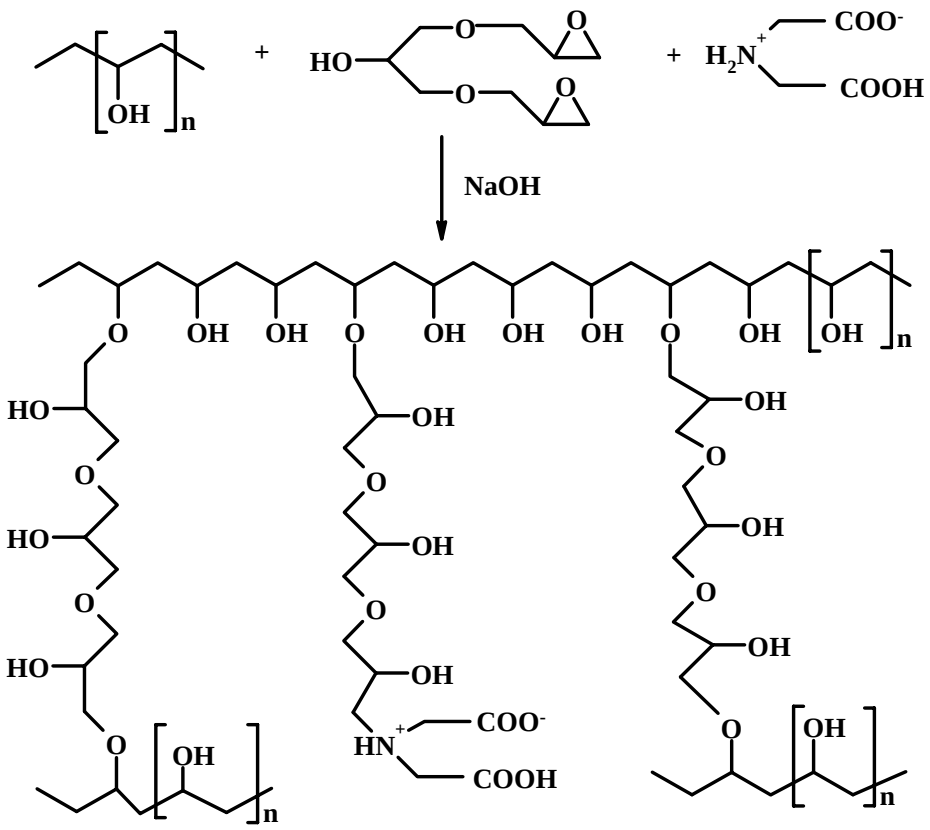


Figure 3. A possible schematic structure for an IDA-PVA hydrogel, showing IDA at its isoelectric point.

Table 3. PVA-based membranes, with the corresponding compounds incorporated in the membrane, and their pI value range.

Membrane	Compound incorporated	pI
IDA-PVA	Iminodiacetic acid	1.7-2.0
ASP-PVA	Aspartic acid	2.0-2.6
GLU-PVA	Glutamic acid	2.6-3.4
QCD-PVA	Quaternary ammonium derivative of β-cyclodextrin	> 11
CDQ-PVA	Quaternary ammonium group in the presence of β-cyclodextrin	> 11
Q-PVA	Quaternary ammonium group	> 11

Three high pH buffering membranes were made using the same synthesis as the low pI membranes, except that instead of incorporating amine-containing ampholytes, the high pH membranes contained a quaternary ammonium derivative. The pK_as of the hydroxyl groups on carbohydrates such as β-cyclodextrin are approximately in the >11 range [30]. With the presence of an at least two equivalents of a hydroxyl group with a $10.5 < pK_a < 14$, and also at least one equivalent of a permanent cation such as a quaternary ammonium functionality, one would have an ampholytic substance that buffers at a high pH value. Therefore, high pH QCD-PVA membranes were made by incorporating

quaternary ammonium derivatives of β-cyclodextrin into the PVA matrix using the crosslinker as a tether. CDQ-PVA membranes were made by reacting separately glycidyl trimethylammonium chloride and β-cyclodextrin with the PVA and GDGE. Q-PVA membranes were made by reacting glycidyl trimethylammonium chloride with PVA and then crosslinking with GDGE, without β-cyclodextrin. Q-PVA membranes rely on the high pK_a values of the hydroxyl groups of the PVA strands. All three membranes have pI values above 11. Table 3 lists the membranes, the compound incorporated into the matrix used to set the pI, and the operational pH value of the membrane. These membranes have been successfully used in IET separations of small ampholytic molecules and proteins, and have been able to withstand high current densities, high power loads, and alkaline solutions as strong as 1 M for experiments as long as 12 hours.

2. PVA-BASED BUFFERING MEMBRANES CONTAINING GRAFTED DIAMINOCARBOXYLIC ACIDS*

2.1 Objectives and rationale

High incorporation rates were achieved when a dicarboxylic acid with a primary amine group, glycerol-1,3-diglycidyl ether and poly(vinyl alcohol) were reacted to produce the poly(vinyl alcohol)-based acidic hydrogels [31]. Work on the synthesis of single-component, diaminosulfopropane carrier ampholytes indicated that many diaminocarboxylic acids had pI values in the $6 < pI < 10$ range [37]. Thus, diaminocarboxylic acids (DACAs) containing one or more $-NH_2$ groups were selected as buffering ampholytes to be incorporated into the poly(vinyl alcohol)-based buffering membranes (called DACA-PVA membranes) described in this section. The pK_a values of the diaminocarboxylic acids were obtained from a collection of critical stability constants [53, 54]. It is known that substitution of a primary amine with a 2-hydroxyethyl or 2-hydroxypropyl functional group leads to a higher pK_b value (i.e., a lower pK_a value for the corresponding conjugate acid). Thus, it was postulated that using diaminocarboxylic acids such as 1,3-diaminopropionic acid (DAPA), histidine (HIS), ornithine (ORN), or lysine (LYS), one might be able to produce mechanically and hydrolytically stable membranes that buffer in the $6 < pH < 8.5$ range.

2.2 Synthesis of DACA-PVA membranes

The DACA-PVA membranes that buffer in the 6 < pH < 8.5 range were synthesized using a slightly modified version of the technology described for the preparation of the low-pH PVA-based buffering membranes [31]. A schematic representation of the synthesis is shown in Figure 4. Histidine was used as the ampholyte to produce the HIS-PVA membranes, 1,3-diaminopropionic acid the DAPA-PVA membranes, ornithine the ORN-PVA membranes, and lysine the LYS-PVA membranes.

To make the DACA-PVA membranes, a reaction mixture containing 100 mL deionized water, 12.56 g NaOH and then a weighed amount of the selected ampholyte (resulting in a 160 mM concentration of the corresponding ampholyte) is mixed in a 500 mL, three-neck, round bottom flask fitted with an ice-water cooled condenser, a nitrogen purge line and a Teflon-coated magnetic stir bar. Then, 20 g of PVA is added to the flask, the system is purged with nitrogen, stirred and heated until all of the PVA is dissolved. Ninety grams of the hot, viscous reaction mixture is then quickly, but thoroughly, mixed with 12.98 g of GDGE and applied over both sides of a PVA paper substrate that is sandwiched between two polypropylene sheets. This recipe will yield a membrane sheet or sheets that are about 900 cm^2 in total area. The excess reaction mixture is expelled and the closed mold is kept at room temperature for about 40 hours. The cured membrane is thoroughly rinsed with deionized water. The membrane sheets are stored in deionized water in the refrigerator at 4 ^oC until used. The membranes show no signs

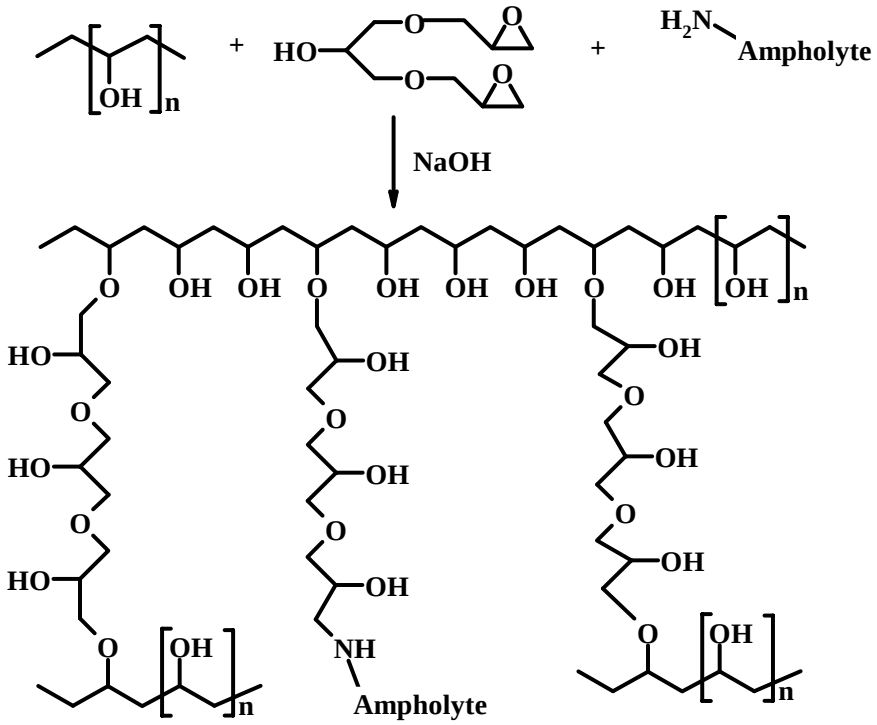


Figure 4. Possible structure for a primary amine-containing ampholyte attached to the PVA hydrogel.

of degradation even when stored in 3 M base for up to 6 months, or in deionized water for up to 12 months. The thickness of the membranes is typically between 0.2 and 1.5 mm. Gloves are worn when the DACA-PVA membranes are handled. The used membranes are disposed as solid waste. Since the recipes yield an ampholyte concentration of 160 mM, the membranes have ample buffering capacity and is - about sixteen times higher than what is needed to reach the limiting pH value of the isoionic solution for the particular ampholyte. Figure 5 shows how ampholyte concentration affects the pH of isoionic solutions of HIS, ORN, DAPA, and LYS, calculated using the PeakMaster Version 5.0 computer program [55-58]. At concentrations of 10 mM, the calculated pH values leveled off to a value equal to the pI of the component. Using an excess amount of ampholyte in the membranes insures that the pH of the membrane remains at the required value, even when the incorporation rate is lower than 100 %.

2.3 Desalting experiments using the DACA-PVA membranes

The DACA-PVA membranes were first tested in IET desalting experiments, in the single separation compartment configuration of the BF200IET shown in Figure 6, using IDA-PVA as the anodic membrane and either HIS-PVA, DAPA-PVA, ORN-PVA or LYS-PVA as the cathodic membrane.

The preparative-scale IET separations were carried out on a BF200IET unit (Life Therapeutics, French's Forest, NSW, Australia) that was modified in our laboratory [19].

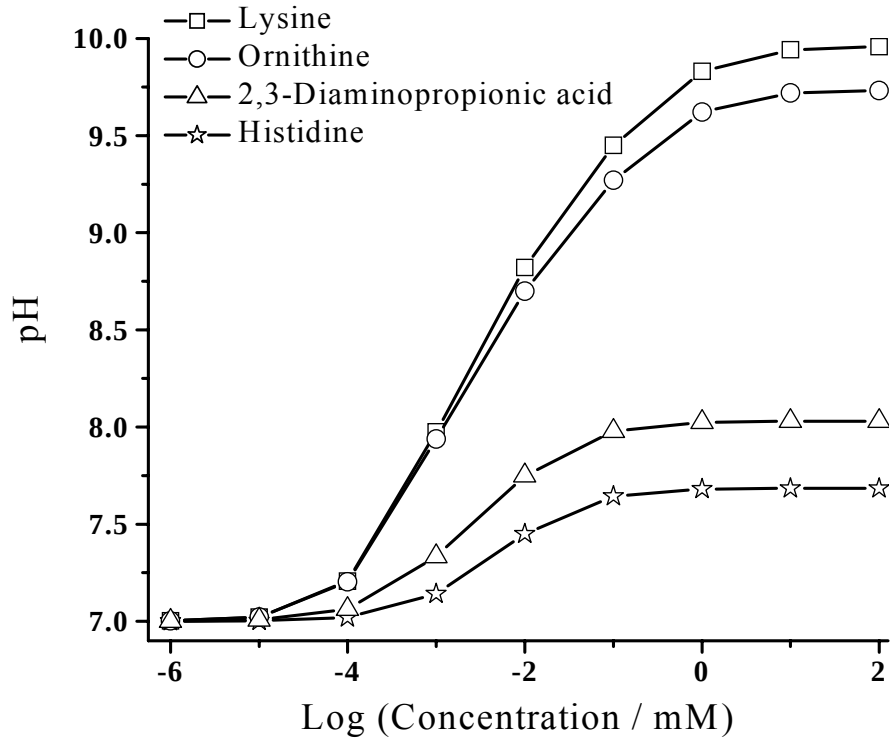


Figure 5. pH as a function of log (concentration) for isoionic solutions of lysine, ornithine, diaminopropionic acid and histidine, calculated by PeakMaster 5.0.

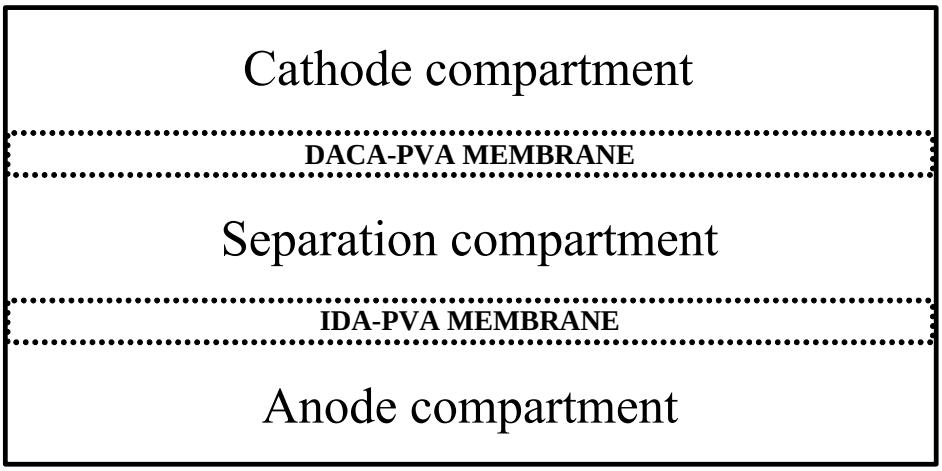


Figure 6. Schematic of the separation head of the BF200IET unit operated in the single separation compartment mode for the trapping and desalting experiments.

A 1000 V, 1200 mA power supply (E-C Apparatus, Co., Holbrook, NY, USA) was used for all IET experiments. The intermembrane distance was about 1 mm and the active membrane surface area was about 15 cm^2. Joule heat was dissipated by recirculating ice water through the glass jackets of the containers that held the anolyte, feed solution, receiving solution and the catholyte. The anodic and cathodic membranes were hydrolytically-stable, PVA-based acidic and alkaline buffering membranes prepared in our laboratory [30-32]. The separation membranes were the PVA-based $6.0 < pH < 8.5$ DACA-PVA membranes described in Section 2.1. IET separations in the BF200IET unit were carried out in the recirculating mode [19], at feed and collection stream flow rates of 30 mL/min, and anolyte and catholyte flow rates of 2 L/min. The applied power loads

were between 30 to 70 W (see the respective experiments). Fractions were collected from all streams at pre-set times and analyzed by CE on a P/ACE 5500 (Beckman-Coulter, CA). The pH values of the samples were measured with a glass microelectrode MI-414 (Microelectrodes, Inc., Bedford, NH, USA) and a Corning Model 150 pH meter (Corning, Inc., Corning, NY, USA). The specific conductivities of the samples were measured with a MI-905 conductivity microprobe (Microelectrodes, Bedford, NH, USA) and a model 145+ conductivity meter (Thermo-Orion, Beverly, MA, USA).

In the desalting experiments, the anolyte contained 30 mM methanesulfonic acid, the catholyte 80 mM NaOH, the sample solution 10 mM benzyltrimethylammonium benzenesulfonate ($BzTMA^+BS^-$, a strong electrolyte salt), 1 mM m-aminobenzoic acid (MABA) and 1 mM 4-hydroxy-3-(morpholinomethyl)-benzoic acid (HMMB). IET was carried out at a constant current of 250 mA (current density = 16.7 mA/cm^2), with typical starting potentials of 20-35 V and finishing potentials of 530-730 V.

Samples were taken from the recirculating solution every 5 min and analyzed by capillary electrophoresis (CE) using a P/ACE 5500 unit (Beckman-Coulter, Fullerton, CA, USA). The UV absorbance detector of the P/ACE was set at 214 nm, the cartridge coolant was thermostated at 25 ^oC. An uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an I.D. of 27 μm, injector-to-detector length of 20 cm and total length of 26 cm was used for the CE analysis of the samples from the trapping and desalting experiments, and another uncoated fused silica capillary

(Polymicro Technologies) with an I.D. of 27 μm, injector-to-detector length of 39 cm and total length of 46 cm was used for the CE analysis of the samples from the membrane pH determination experiments. The background electrolyte (BGE) was a 20 mM boric acid solution, titrated to pH=9.0 with LiOH. A potential of 15 kV was applied at the injector end of the capillary.

The electropherograms for the 0 min (feed), 10 min and 40 min samples in the desalting experiment using DAPA-PVA are shown in the top, middle and bottom panels, respectively, of Figure 7. The strong electrolytes are completely removed by 40 min and neither MABA nor HMMB were lost during the course of the run. The IET current was set at 250 mA. The specific conductivity, pH values of the recirculating solution, and IET potential are shown as a function of time in Figure 8. $BzTMA^+$ is removed at a faster rate than BS^- hence, in the first part of the desalting process, the pH of the recirculating solution temporarily becomes lower than its initial value [46]. By the end of the run, the pH increases back to its stable, final value.

The desalting experiments with HIS-PVA, ORN-PVA, and LYS-PVA behaved in a similar manner as DAPA-PVA. With HIS-PVA, the strong electrolytes were removed in 50 min, as seen in Figure 9. The specific conductivity, pH of the recirculating solution, and IET potential are graphed as a function of time in Figure 10. Similar electropherograms and graphs for ORN-PVA and LYS-PVA are shown in Figures 11-14. As with DAPA-PVA, $BzTMA^+$ is removed at a faster rate than BS^- hence, in the

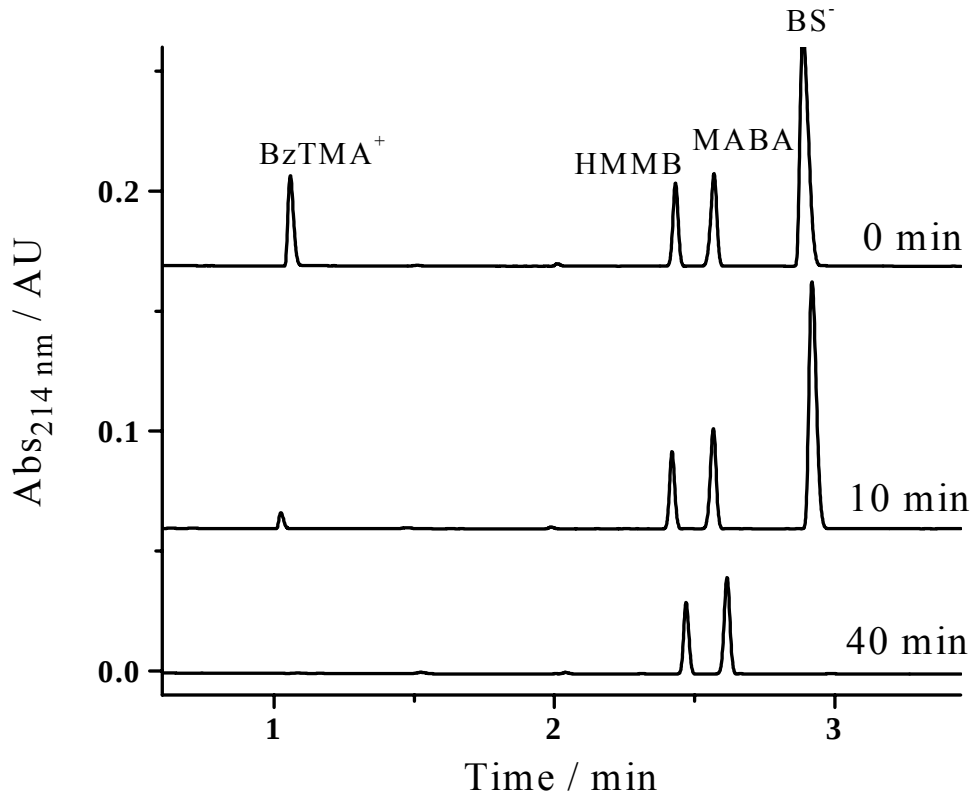


Figure 7. Electropherograms of the samples taken from the IET desalting experiment with DAPA-PVA as the cathodic membrane. Top panel: feed (0 min) sample; middle panel: 10 min sample; bottom panel: 40 min sample.

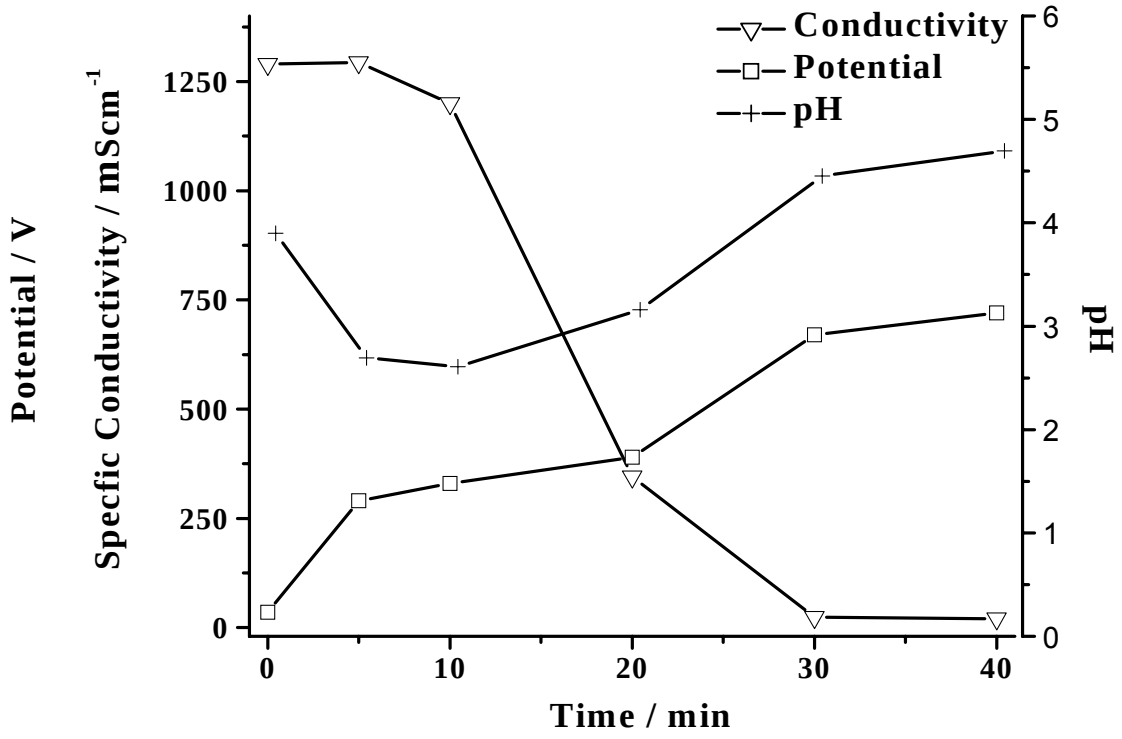


Figure 8. Potential during the experiment, and specific conductivity and pH of the recirculated solution during IET desalting with DAPA-PVA as the cathodic membrane. IET current: 250 mA.

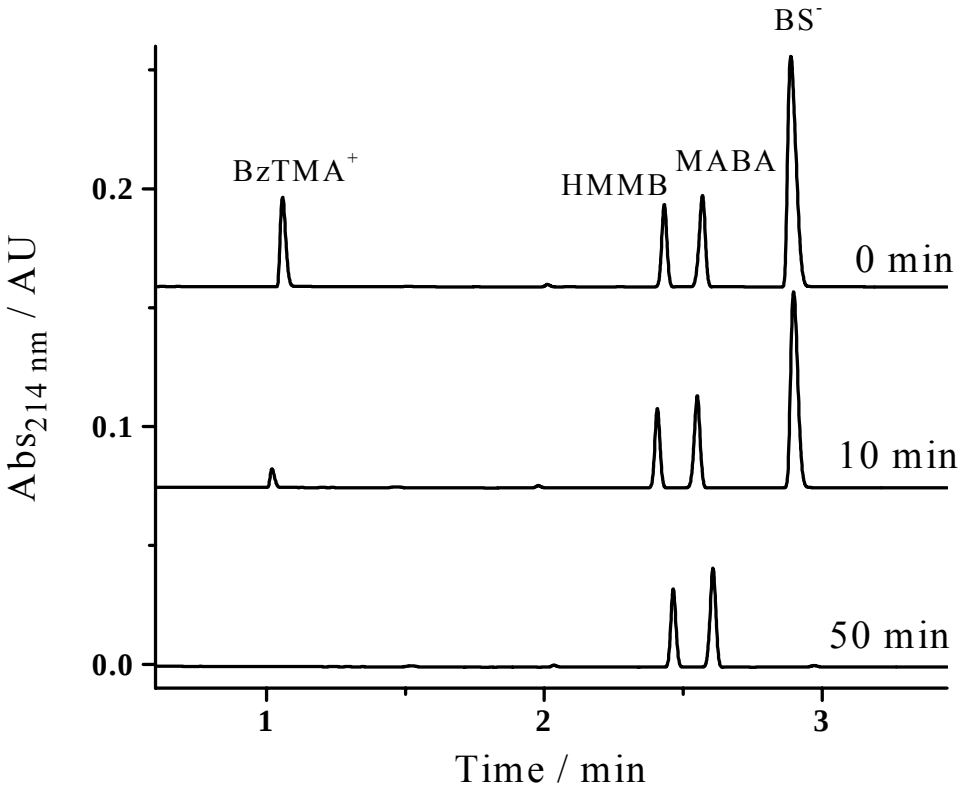


Figure 9. Electropherograms of the samples taken from the IET desalting experiment with HIS-PVA as the cathodic membrane. Top panel: feed (0 min) sample; middle panel: 10 min sample; bottom panel: 50 min sample.

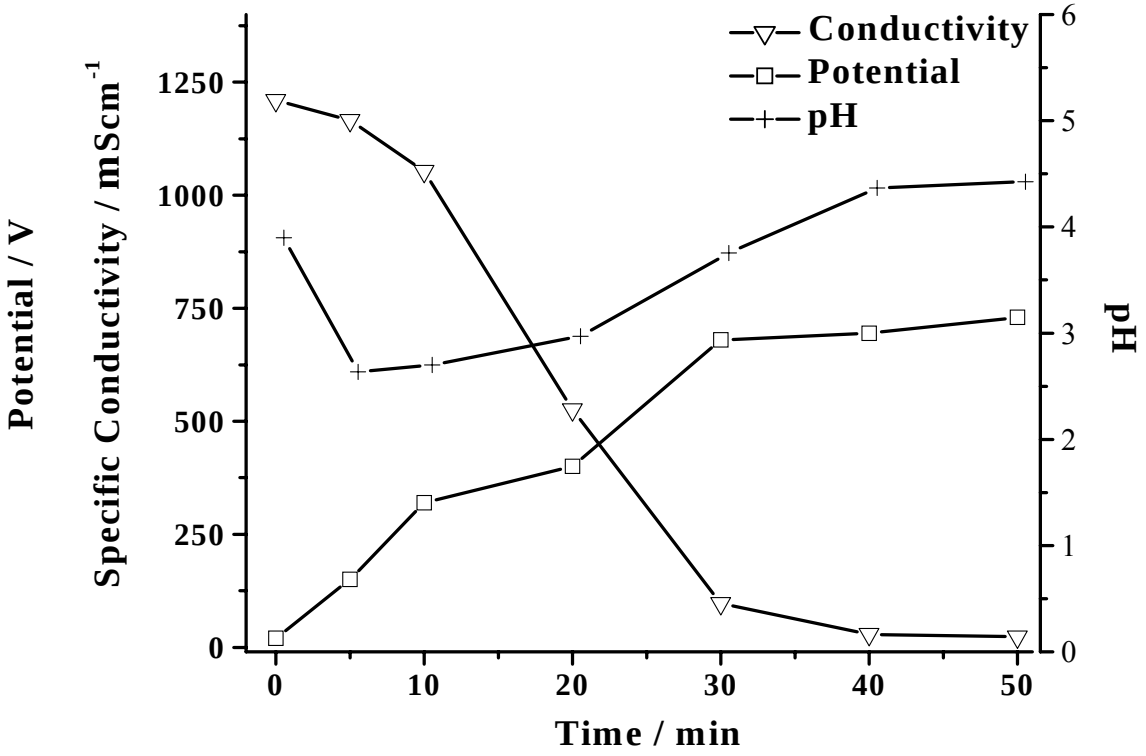


Figure 10. Potential during the experiment, and specific conductivity and pH of the recirculated solution during IET desalting with HIS-PVA as the cathodic membrane. IET current: 250 mA.

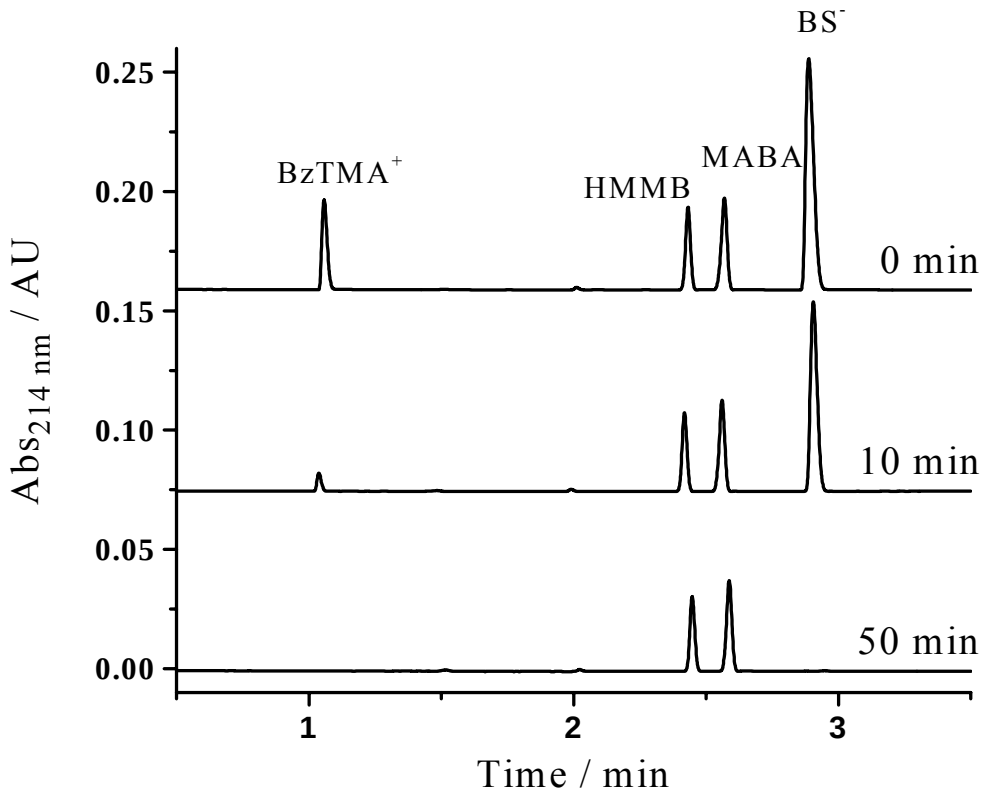


Figure 11. Electropherograms of the samples taken from the IET desalting experiment with ORN-PVA as the cathodic membrane. Top panel: feed (0 min) sample; middle panel: 10 min sample; bottom panel: 50 min sample.

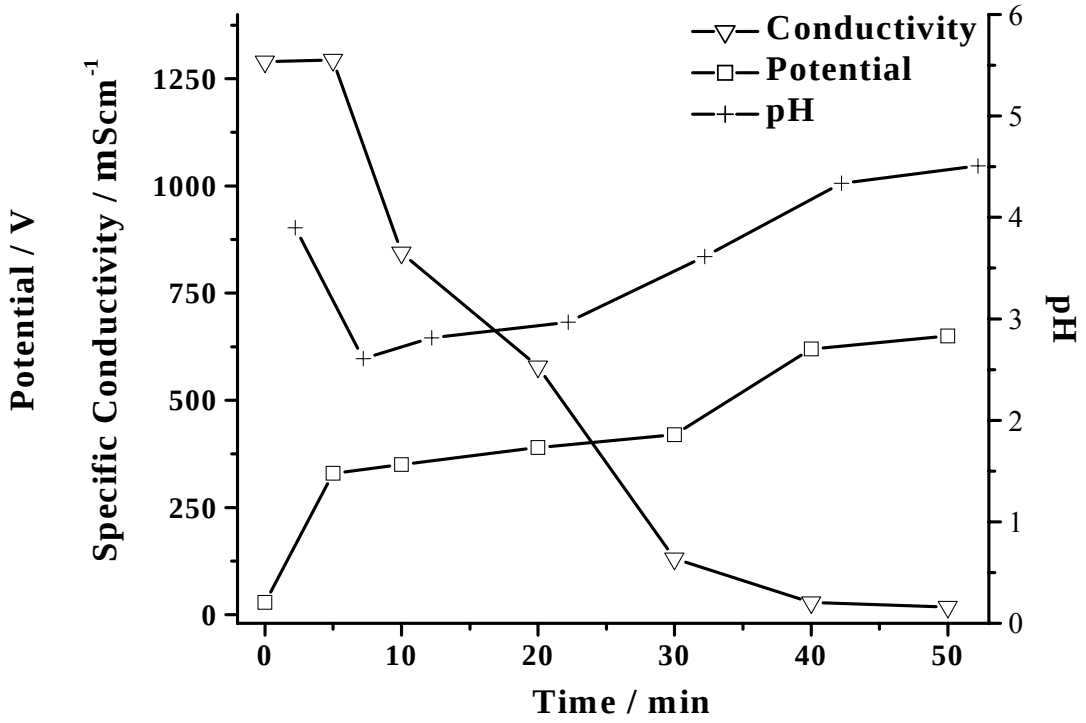


Figure 12. Potential during the experiment, and specific conductivity and pH of the recirculated solution during IET desalting with ORN-PVA as the cathodic membrane. IET current: 250 mA.

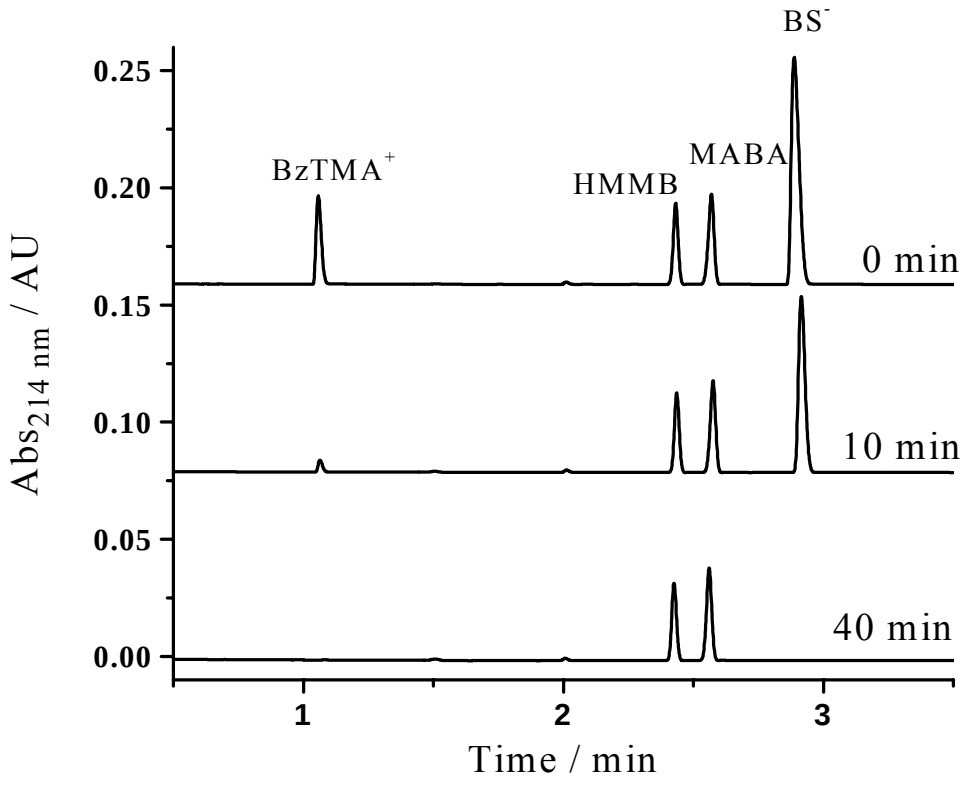


Figure 13. Electropherograms of the samples taken from the IET desalting experiment with LYS-PVA as the cathodic membrane. Top panel: feed (0 min) sample; middle panel: 10 min sample; bottom panel: 40 min sample.

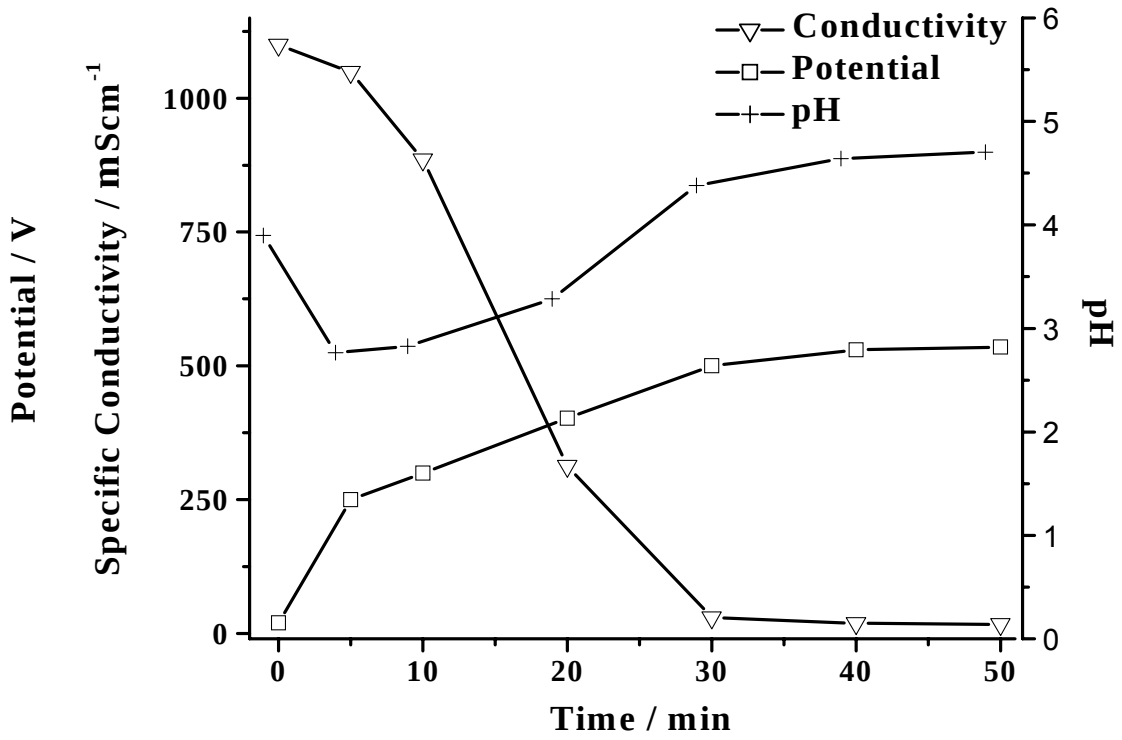


Figure 14. Potential during the experiment, and specific conductivity and pH of the recirculated solution during IET desalting with LYS-PVA as the cathodic membrane. IET current: 250 mA.

first part of the desalting process, the pH of the recirculating solution temporarily becomes lower than its initial value, but returns to its final value by the end of the run.

2.4 Testing of the DACA-PVA buffering membranes in isoelectric trapping mode

The new, middle-pH DACA-PVA membranes were next tested as cathodic membranes in the BF200IET using the same preparative-scale IET procedure as for the desalting experiments (operated in the single separation compartment configuration, shown schematically in Figure 6) to see if they could trap ampholytic analytes. The anodic buffering membrane was an IDA-PVA membrane [31]. The anolyte contained 40 mM methanesulfonic acid and 3 mM benzenesulfonic acid. The catholyte contained 80 mM NaOH and 2.5 mM benzyltrimethylammonium hydroxide. The UV-absorbing benzenesulfonic acid and benzyltrimethylammonium hydroxide allowed us to monitor if there was any unwanted intrusion of the anolyte or catholyte into the separation compartment. A solution of 1 mM *m*-aminobenzoic acid (MABA, approximate pI = 3.9 [53]), and 1 mM 4-hydroxy-3-(morpholinomethyl)-benzoic acid (HMMB, approximate pI = 5.9) was used for the experiments with the DAPA-PVA and HIS-PVA membranes. MABA (1 mM) and 2 mM histidine (HIS, approximate pI = 7.6) was used for the experiment with the ORN-PVA membrane. One mM MABA, 2 mM HIS, and 2 mM carnosine (CAR, approximate pI = 8.1 [53]) were used as ampholytic analytes in the isoelectric trapping experiments with the LYS-PVA membrane. Electrophoresis was continued in recirculating mode for 3 h in all experiments. Aliquots were collected from the recirculating solution every half hour and analyzed by CE, using the same

experimental procedures as for the desalting experiments. A constant potential of 250 V was applied in all of the isoelectric trapping experiments. This potential generated a typical initial IET current of 210-250 mA, the current dropped to 50-60 mA within 5-10 min and remained constant for the duration of the run (corresponding to a current density of approximately 3.3-4.0 mA/cm^2).

Figure 15 shows the results of the CE analysis for the starting sample (top panel), 1 h sample (middle panel) and the 3 h sample (bottom panel) using DAPA-PVA as the cathodic membrane. MABA and HMMB were trapped for the duration of the 3 h run, and neither benzenesulfonate (BS^-) nor benzyltrimethylammonium ($BzTMA^+$) invaded the separation compartment. Figures 16-18 show the electropherograms for the trapping experiments with HIS-PVA, ORN-PVA, and LYS-PVA, respectively. As can be seen, none of the ampholytes were lost during IET, and the strong electrolytes did not invade the separation compartments.

2.5 Determination of the pH value ranges of the DACA-PVA membranes

The effective pH values of the new, DACA-PVA membranes were determined under actual operating conditions, by using them as separation membranes in the two separation compartment configuration of the BF200IET (Figure 19). The anodic and cathodic membrane in each case was an acidic IDA-PVA and a basic Q-PVA membrane, respectively. The anolyte was a 30 mM methanesulfonic acid solution, the catholyte a

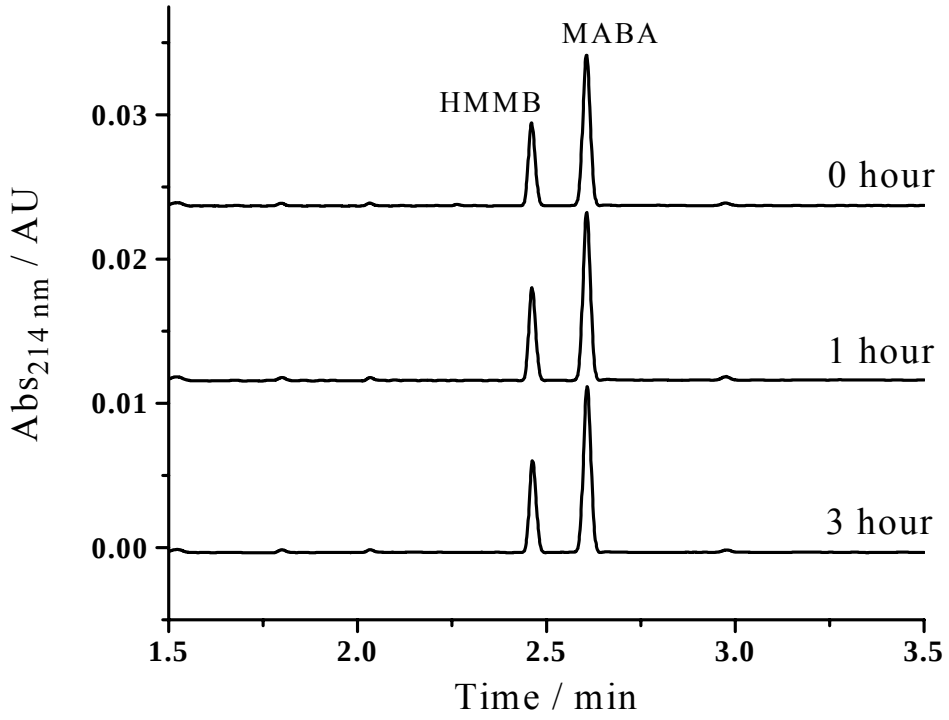


Figure 15. Electropherograms of the samples taken from the IET experiments with DAPA-PVA as the separation membrane. Top panel: feed (0 min) sample; middle panel: 1 h sample; bottom panel: 3 h sample.

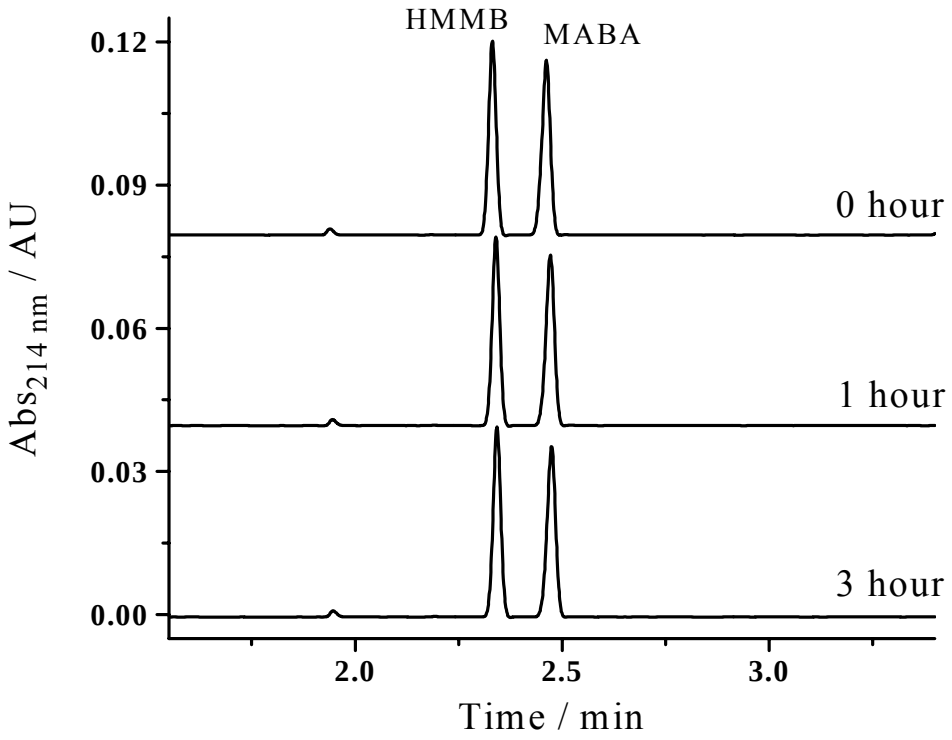


Figure 16. Electropherograms of the samples taken from the IET experiments with HIS-PVA as the separation membrane. Top panel: feed (0 min) sample; middle panel: 1 h sample; bottom panel: 3 h sample.

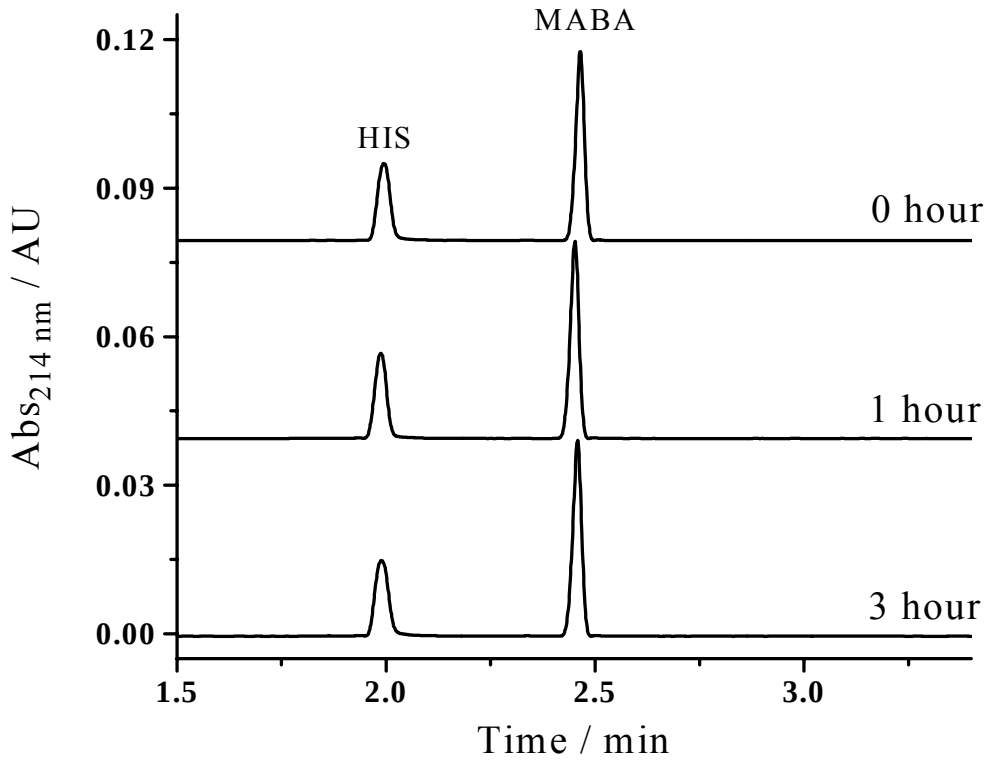


Figure 17. Electropherograms of the samples taken from the IET experiments with ORN-PVA as the separation membrane. Top panel: feed (0 min) sample; middle panel: 1 h sample; bottom panel: 3 h sample.

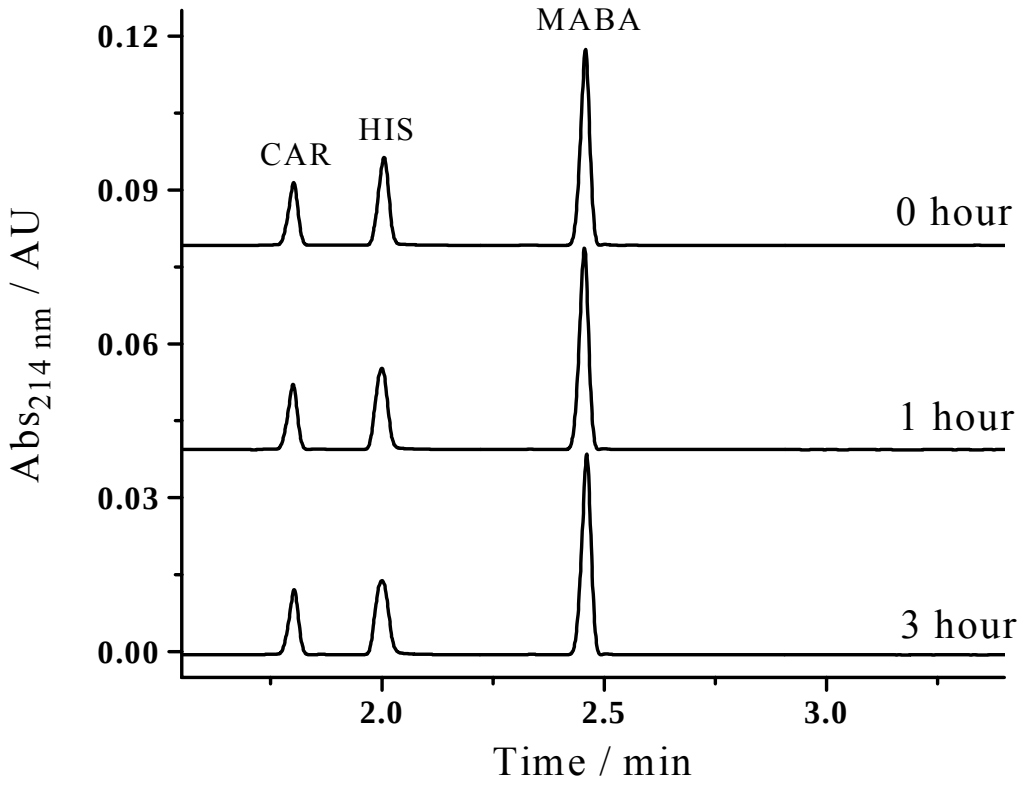


Figure 18. Electropherograms of the samples taken from the IET experiments with LYS-PVA as the separation membrane. Top panel: feed (0 min) sample; middle panel: 1 h sample; bottom panel: 3 h sample.

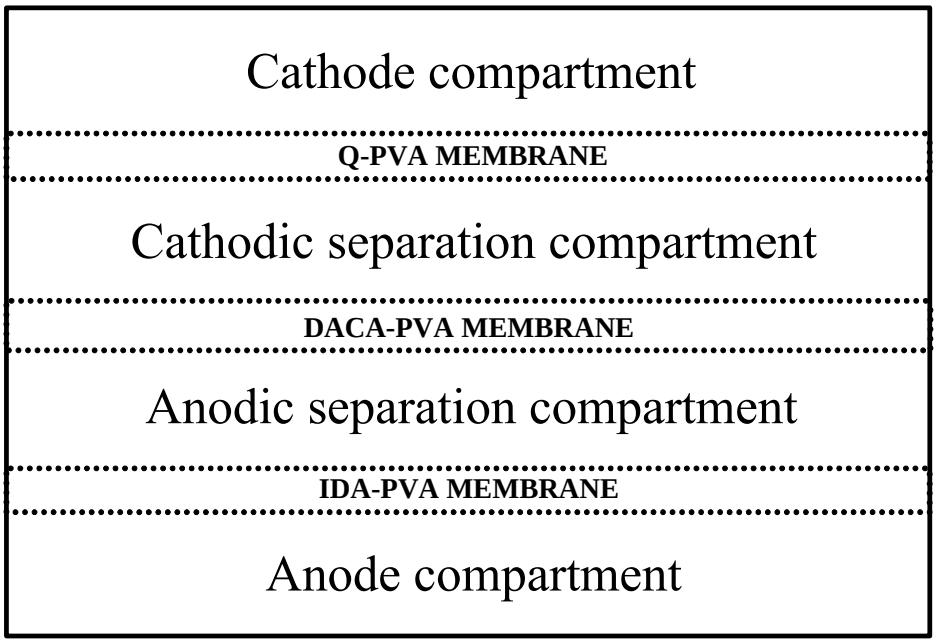


Figure 19. Schematic of the separation head of the BF200IET unit operated in the two separation compartment mode for the membrane pH determination and chicken egg white protein separation experiments.

180 mM NaOH solution. Both the anodic and cathodic separation compartments were filled with a solution that contained pI markers whose pI values were as close to the expected pH of the DACA-PVA separation membrane as possible. The pI markers were dissolved in deionized water and their concentrations were in the 1 to 2 mM range. The IET separations were carried out at 300 V, in constant potential mode, that resulted in typical initial currents of 90 to 120 mA and finishing currents of 75 to 85 mA. The currents typically leveled off in 30 to 50 min as the ampholytes were moved to their respective final compartments. However, in order to ascertain that the pH of the DACA-PVA membranes did not change during an extended IET experiment, the IET runs were continued for an hour and a half. The width of the pH range listed depends on the availability of appropriate pI markers, for some of the DACA-PVA membranes it is as small as 0.2, for others as large as 0.4. In order to narrow the pH range when appropriate, closely spaced, small-molecule pI markers were not available, such as for the HIS-PVA and DAPA-PVA membranes, the limiting pH values were further narrowed based on the results of the IET egg white separation experiments (*vide infra*).

The electropherograms of the samples taken from the membrane pH determination IET experiment with LYS-PVA as the separation membrane are shown in Figure 20. The feed (0 min) sample is shown in the top panel, the content of the cathodic separation compartment after 90 min in the middle panel, and the content of the anodic separation compartment after 90 min in the bottom panel. Carnosine (CAR, approximate pI = 8.1) ends up in the anodic separation compartment, labetalol (LAB, approximate pI = 8.3) in

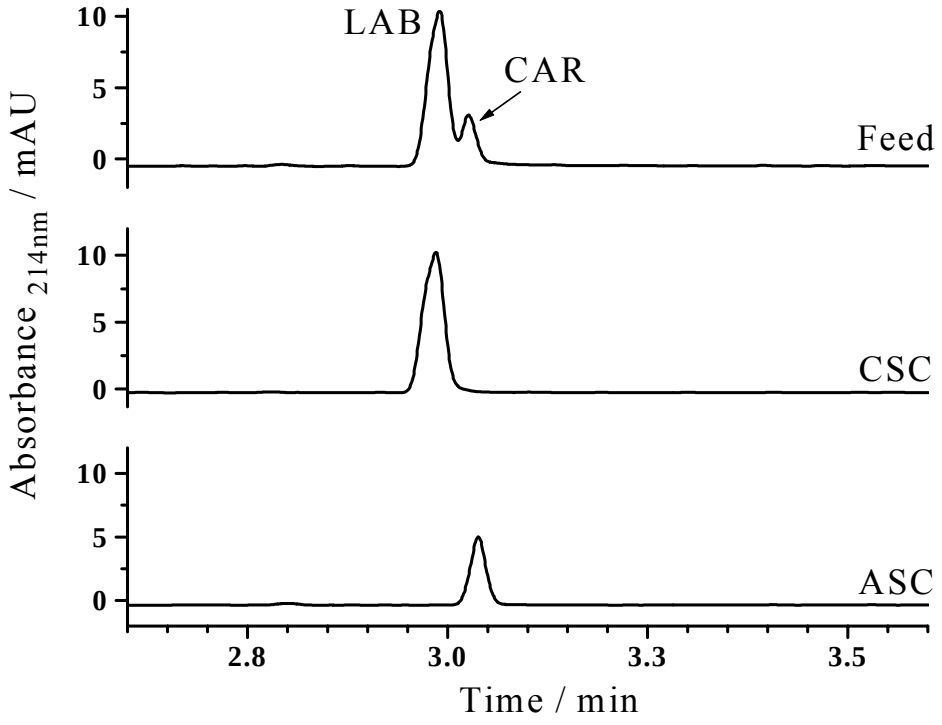


Figure 20. Electropherograms of the samples taken from the membrane pH determination IET experiment with LYS-PVA as the separation membrane. Top panel: feed solution; middle panel: cathodic separation compartment (CSC); bottom panel: anodic separation compartment (ASC).

the cathodic separation compartment, therefore the operational pH range for the LYS-PVA membrane is 8.1 < membrane pH < 8.3. The HIS-PVA, ORN-PVA and DAPA-PVA membranes were tested in a similar manner: the appropriate pI markers always ended up in the respective compartments (for HIS-PVA and DAPA-PVA: 4-hydroxy-3-(dimorpholinomethyl)-benzoic acid (approximate pI = 6.3) in the anodic separation compartment, HIS (approximate pI = 7.6) in the cathodic separation compartment; for ORN-PVA: HIS (approximate pI = 7.6) in the anodic separation compartment, CAR (approximate pI = 8.1) in the cathodic separation compartment) and remained there for the duration of the 90 min runs.

2.6 IET separation of chicken egg white proteins using DACA-PVA membranes

Finally, to test if the new, DACA-PVA membranes were suitable for protein separations as well, the major proteins in egg white were separated using the BF200IET, in recirculating mode, in the two separation compartment configuration shown in Figure 19.

The feed solution contained 5 mM lysine as a pH-biaser[20] and chicken egg white as the protein source (diluted to a ratio of 1 mL egg white in 20 mL of the 5 mM lysine solution). The collection stream contained 10 mM glutamic acid. The anolyte was a 30 mM methanesulfonic acid solution, the catholyte a 180 mM sodium hydroxide solution. The anodic membrane was a low pH IDA-PVA membrane, the cathodic membrane a high pH Q-PVA membrane.

Fractions were collected from the feed and collection streams every 15 min and analyzed by the PA 800 system (Beckman-Coulter, Fullerton, CA) using the Proteome Lab™ SDS-MW Analysis kit, Part Number 390953 (Beckman-Coulter, Fullerton, CA) and the associated protocol [59]. The capillary (100 μm I.D., 19 cm injector-to-detector length, 29 cm total length) was filled with the Proteome Lab™ SDS-MS Gel Buffer (Beckman-Coulter). The samples were prepared by mixing 150 μL of the fractions collected in the IET run with 50 μL of the Proteome Lab™ SDS-MW Sample Buffer (Beckman-Coulter), 4 μL of the Proteome Lab™ Internal Standard (approximate relative molecular mass 10,000), and 10 μL of mercaptoethanol. The mixtures were heated in a water bath at 100 ^{o}C for 3 min, cooled to room temperature in a water bath for 5 min, and injected for 20 s by 5kV. The separations were carried out for 30 min at 15 kV, at 25 ^{o}C, and the proteins were detected at 220 nm.

The electropherograms of the samples taken from the experiment conducted with the HIS-PVA membrane as the separation membrane are shown in Figure 21, and for DAPA-PVA as the separation membrane in shown in Figure 22. The feed (0 min) sample is shown in the top panel, the content of the cathodic separation compartment at the end of the IET run (60 min) in the middle panel, and the content of the anodic separation compartment at the end of the IET run (60 min) in the bottom panel. Clearly, there is no cross-contamination between the contents of the two separation compartments, the HIS-PVA and DAPA-PVA membranes performed as expected. Since the isoforms of ovotransferin were trapped in the cathodic separation compartment, the

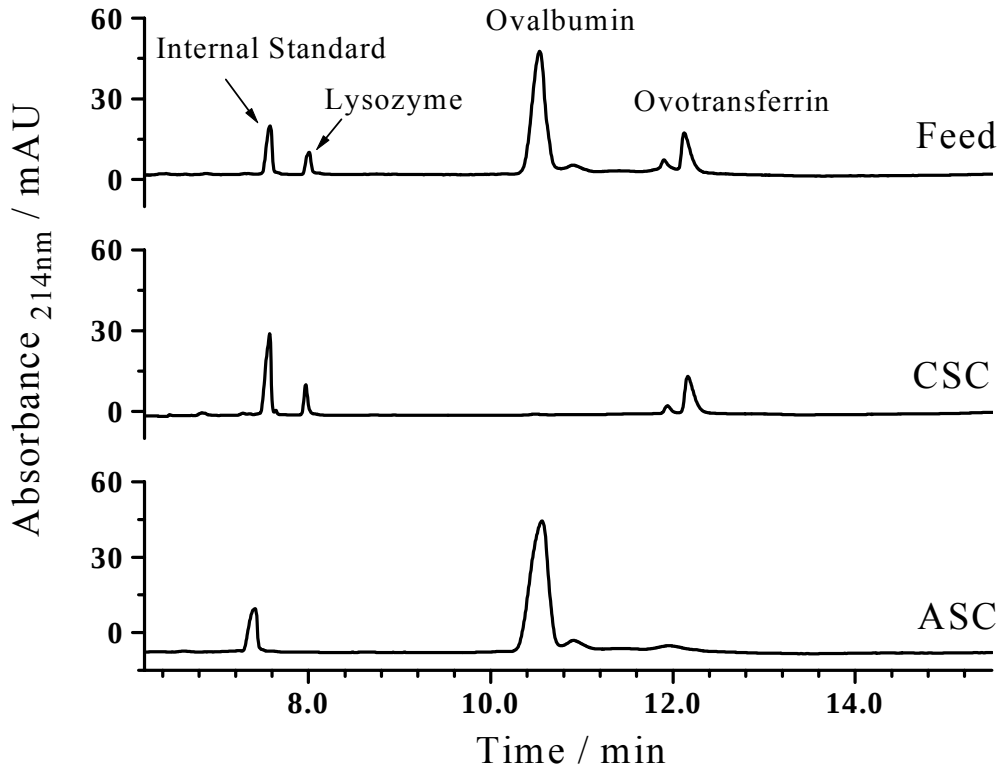


Figure 21. Electropherograms of the samples taken from the IET chicken egg white separation experiment with HIS-PVA as the separation membrane. Top panel: feed solution; middle panel: cathodic separation compartment (CSC); bottom panel: anodic separation compartment (ASC).

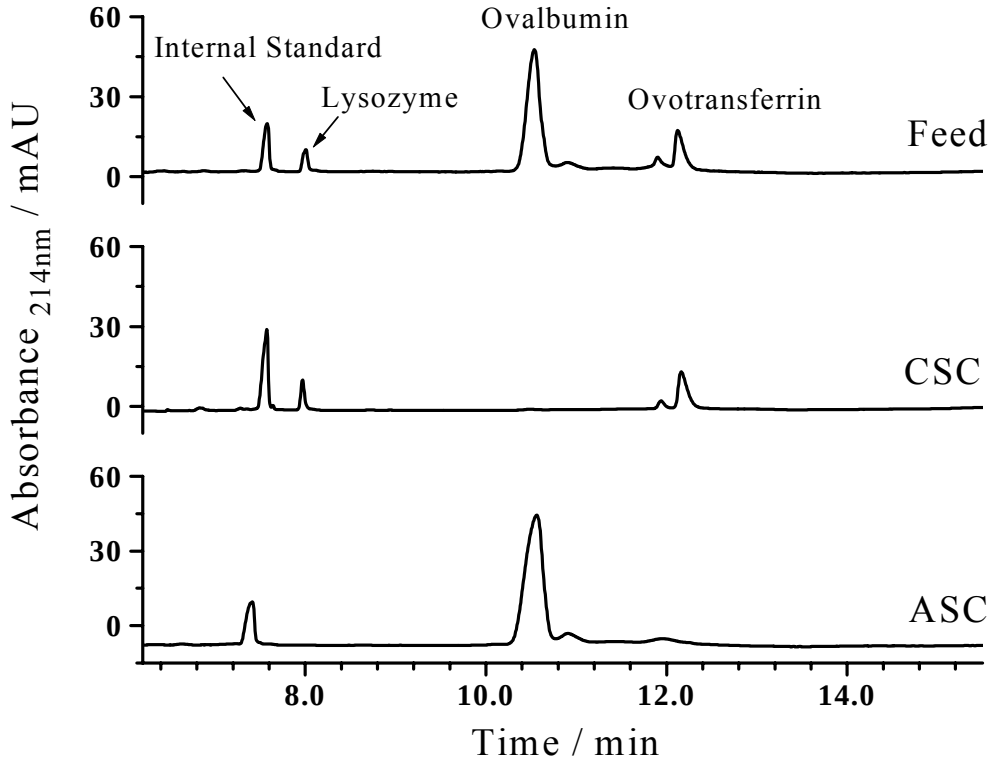


Figure 22. Electropherograms of the samples taken from the IET chicken egg white separation experiment with DAPA-PVA as the separation membrane. Top panel: feed solution; middle panel: cathodic separation compartment (CSC); bottom panel: anodic separation compartment (ASC).

operational pH value of the HIS-PVA and DAPA-PVA membrane has to be below 6.6 [60], narrowing the pH range for these two membranes to 6.3 < membrane pH < 6.6.

3. PVA-BASED BUFFERING MEMBRANES WITH TUNABLE pH VALUES

3.1 Objectives and rationale

One drawback of the PVA-based membranes described in Section 2 is that their pH values can only be tuned stepwise, by selecting a different ampholyte for each membrane. This is not a major drawback if the membranes are to be used as anodic or cathodic membranes, but presents problems if they are to be used as separation membranes. Therefore, the pH of the separation membrane has to be continuously tunable, similarly to that of the acrylamide - Immobiline membranes.

3.2 Preparation of PVA membranes with tunable pH values

In order to synthesize buffering membranes with tunable pH values, it is necessary to choose an appropriate acid and base that contain a functional group which allows attachment to the PVA matrix, and guaranties similar incorporation rates for both the acid and the base. Such a functional group could be a primary amine group. The concentrations of the acid and the base can then be adjusted to reach a desired pH value in the membrane according to the Henderson-Hasselbach equation. Figure 23 shows a representation of a possible hydrogel structure, with acidic and basic functionalities attached to the PVA backbone. These attached acidic and basic functionalities will form an immobilized buffer system (act as the buffering compound and the titrant) and determine the pH in the membrane.

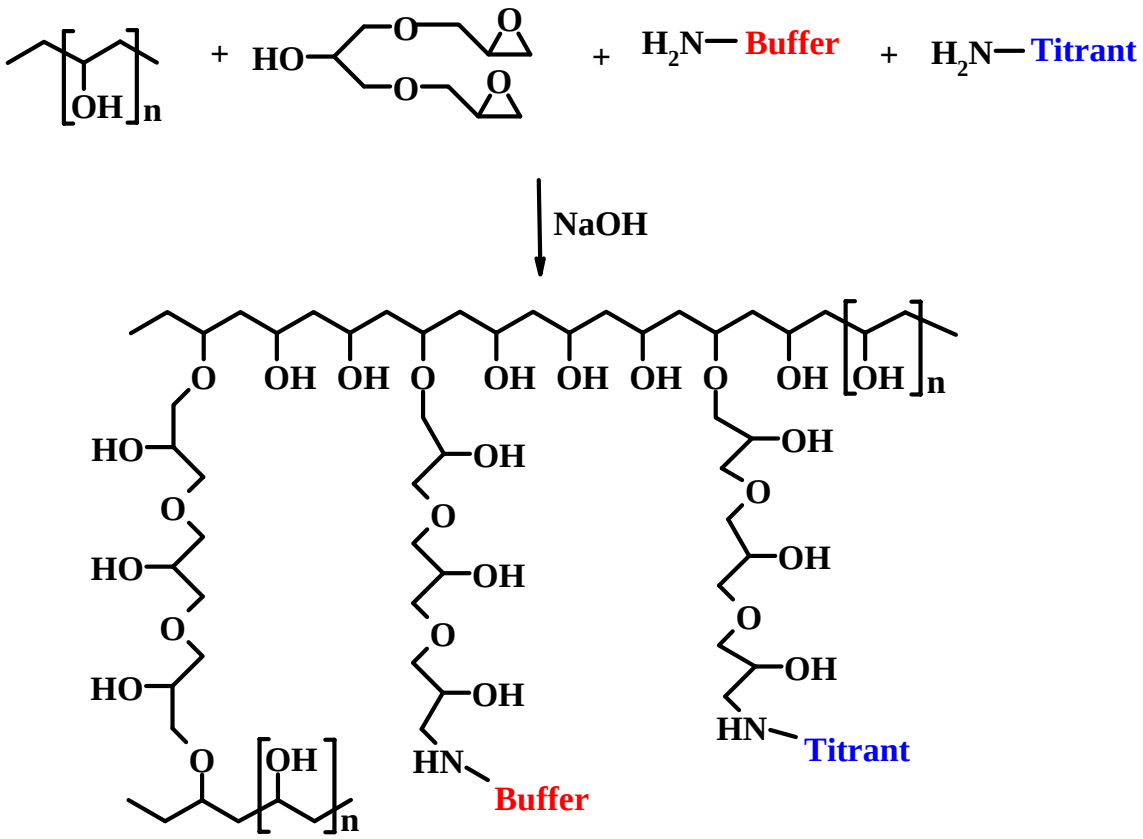


Figure 23. Schematic representation of the synthesis of a PVA-based buffering membrane with tunable pH wherein the buffering and titrating compounds are attached separately.

3.2.1 Synthesis of tunable PVA membranes

A series of primary amino group-containing buffers and titrants were chosen to synthesize PVA membranes that buffer in the range of pH 3 to 10. Two series were chosen to cover the low pH range, two for the mid pH range and two for the high pH range. From work done with the single component, low-pH membranes [31], it was apparent that aminodicarboxylic acids produce good, low-pH buffering membranes. Therefore, aminomalonic acid (MAL), an aminodicarboxylic acid, was selected as the buffering compound to make a series of buffering membranes with low, tunable pH values. The pK_a values of aminomalonic acid are 1.8 and 2.94 [53], therefore it is reasonable to assume that the pK_a values of the carboxylic acid groups of the membrane-bound aminomalonic acid derivative will be low as well. Since aminomalonic acid contains both a primary amine group and two carboxylic acid groups, it can be attached to the PVA matrix via the glycerol diglycidyl ether crosslinker. Though one equivalent of the carboxylic acid groups of aminomalonic acid will be deprotonated by the amino group of aminomalonic acid, the second equivalent can be deprotonated in a controlled manner using an appropriate basic titrant. 3-Amino-1-propanol (PROP) was selected as the basic titrant for these membranes. The pH of this buffer system can be varied by adjusting log (c_{base} / c_{acid}), where c_{base} is the concentration of the COO^- form and c_{acid} is the concentration of the COOH form of the second equivalent of the acidic functional groups of aminomalonic acid. Since only the hydrochloride salt of the diethyl ester form of aminomalonic acid, diethylaminomalonate hydrochloride, is available commercially, this compound was used to make the

membranes: the ester will be automatically hydrolyzed in the basic reaction medium used for the preparation of the membrane. Membranes made with MAL as the source of the buffering compound and PROP as the titrant are called MAL-PROP-PVA membranes.

Another series of buffering membranes with low, tunable pH values was made using aspartic acid (ASP) as the buffering compound. The pK_a values of the carboxylic acid groups of ASP are 2.00 and 3.70 [53]; therefore if a titrant were added to an ASP-PVA membrane in a specified ratio, similarly to what was done with MAL, a less acidic buffering membrane could be made. Buffering membranes made with ASP as the buffering compound and PROP as the titrant are called ASP- PROP-PVA membranes.

The conjugate acids of compounds such as morpholine and N-alkylmorpholine derivatives tend to have pK_a values in the 5 to 9 range (morpholine has a pK_a of 8.58, and N-methylmorpholine has a pK_a of 7.58 [53]). A morpholine derivative was therefore selected as the buffering compound to synthesize a series of buffering membranes whose pH is tunable in the slightly acidic to neutral range. 4-(2-aminoethyl)morpholine (MORPH) was used as the buffering compound, ASP as the titrant. The amount of ASP added was calculated such that buffering would occur around the pK_a of the stronger conjugate acid of MORPH. These buffering membranes are called MORPH-ASP-PVA membranes.

Tris(hydroxymethyl)aminomethane, TRIS, is another amine whose conjugate acid has a pK_a value in the mid-pH range (pK_a = 8.09 [53]). TRIS, therefore, was used as the buffering compound in another series of membranes that had tunable pH in the neutral to slightly basic range. ASP was again used as the titrant. These membranes are called TRIS-ASP-PVA membranes.

Buffering membranes with tunable, high pH values were made with a hydrophilic amine, 3-amino-1-propanol (PROP). Its conjugate acid has a high pK_a value, approximately 10.1 [53]. ASP was again used as the titrant, and was added in appropriate amounts so that buffering would be provided by PROP. These membranes are called PROP-ASP-PVA membranes.

By studying the pK_a values of the conjugate acids of diamino propanes [2], it was found that they differ by about 2, and are in the $7 < pK_a < 11$ range. Therefore, 3-(diethylamino)propylamine (DEAPA) was selected as the buffering compound to make another series of membranes with tunable, high pH values. Aspartic acid (ASP) was used again as the titrant. The amount of ASP added was calculated to yield a buffer that utilized the weakest conjugate acid in the system. These membranes are called DEAPA-ASP-PVA membranes**.** Table 4 lists the six different membrane series that were synthesized, their expected pH range, and the buffer and titrant used in their synthesis.

Table 4. The six different membrane series, and the buffer and titrant used in the syntheses of these PVA-based buffering membranes.

Membrane	Expected pH Range	Buffer	Titrant
MAL-PROP-PVA	Low	Aminomalonic acid	3-Amino-1-propanol
ASP-PROP-PVA	Low	Aspartic acid	3-Amino-1-propanol
MORPH-ASP-PVA	Mid	4-(2-Aminoethyl)morpholine	Aspartic acid
TRIS-ASP-PVA	Mid	Tris(hydroxymethyl)amino-methane	Aspartic acid
PROP-ASP-PVA	High	3-Amino-1-propanol	Aspartic acid
DEAPA-ASP-PVA	High	3-(Diethylamino)propylamine	Aspartic acid

All of the buffering membranes were synthesized using a slightly modified version of the technology described for the preparation of the low and mid-pH, single component PVA-based buffering membranes[31, 32]. Briefly, a reaction mixture containing a calculated amount of water, sodium hydroxide, and the calculated amount of buffer and titrant were added to a 500 mL round bottom flask that was fitted with an ice-water cooled condenser, a nitrogen purge line, and a Teflon-coated stir bar. The solution was stirred until all of the chemicals have dissolved. Next, a calculated amount of PVA was added to the round bottom flask. The reaction mixture was stirred and heated until all of the PVA has dissolved. Then, 90 g of the hot reaction mixture was weighed out and mixed with a calculated amount of the crosslinker, GDGE. This was then poured over

both sides of a PVA paper substrate that was sandwiched between two polypropylene sheets. The excess reaction mixture was then expelled from the sheets using a Teflon coated rolling pin. The membranes were allowed to cure for 40 hours, at room temperature, in a closed container, and were then rinsed with, and stored in, deionized water until used. The membranes showed no signs of degradation even when stored in 3 M NaOH for up to 3 months. Gloves were worn at all times when handling the membranes, and the used buffering membranes were disposed of as solid waste. The volume of water and the amounts (in grams) of sodium hydroxide, buffer, titrant, PVA and GDGE used for a specified membrane composition, corresponding to a specific log (c_{base} / c_{acid}), are listed in Tables 5-10 for the MAL-PROP-PVA, ASP-PROP-PVA, MORPH-ASP-PVA, TRIS-ASP-PVA, PROP-ASP-PVA, and DEAPA-ASP-PVA membranes, respectively. The amounts listed are used for a PVA paper substrate that has an area of 900 cm^2.

3.3 Membrane pH determination

In order to characterize the PVA-based buffering membranes that have tunable pH values, their pH values were determined for a range of buffer and titrant ratios. For each membrane series, several membranes were synthesized whose pH differed by about 0.1 (or less, for some of the experiments). Each buffering membrane was then tested in a preparative-scale IET separation using small ampholytic molecules (pI markers) as probes. The preparative-scale IET separations were carried out on a BF200IET unit (Life Therapeutics, Frenchs Forest, NSW, Australia) that was modified in our laboratory

Table 5. Recipes for the preparation of MAL-PROP-PVA membranes.

Log (c_{base} / c_{acid})	Volume H_20 / mL	Mass NaOH / g	Mass MAL / g	Mass PROP / g	Mass PVA / g	Mass GDGE / g per 90 g reaction mixture
1.5	100	12.56	3.432	1.181	20.0	12.78
1.0	100	12.56	3.432	1.107	20.0	12.78
0.9	100	12.56	3.432	1.082	20.0	12.78
0.7	100	12.56	3.432	1.015	20.0	12.79
0.6	100	12.56	3.432	0.973	20.0	12.79
0.5	100	12.56	3.432	0.925	20.0	12.80
0.4	100	12.56	3.432	0.871	20.0	12.80
0.3	100	12.56	3.432	0.811	20.0	12.81
0.2	100	12.56	3.432	0.747	20.0	12.81
0.1	100	12.56	3.432	0.679	20.0	12.82
0.0	100	12.56	3.432	0.609	20.0	12.82
-0.1	100	12.56	3.432	0.539	20.0	12.83
-0.4	100	12.56	3.432	0.347	20.0	12.85
-0.5	100	12.56	3.432	0.293	20.0	12.85
-0.6	100	12.56	3.432	0.245	20.0	12.86
-0.7	100	12.56	3.432	0.203	20.0	12.86
-0.9	100	12.56	3.432	0.136	20.0	12.87
-1.0	100	12.56	3.432	0.111	20.0	12.87

Table 6. Recipes for the preparation of ASP-PROP-PVA membranes.

Log (c_{base} / c_{acid})	Volume H_20 / mL	Mass NaOH / g	Mass ASP / g	Mass PROP / g	Mass PVA / g	Mass GDGE / g per 90 g reaction mixture
1.30	100	12.56	2.158	1.160	20.0	12.90
1.20	100	12.56	2.158	1.146	20.0	12.90
0.95	100	12.56	2.158	1.095	20.0	12.90
0.80	100	12.56	2.158	1.051	20.0	12.91
0.60	100	12.56	2.158	0.973	20.0	12.92
0.50	100	12.56	2.158	0.925	20.0	12.92
0.25	100	12.56	2.158	0.780	20.0	12.93
0.20	100	12.56	2.158	0.747	20.0	12.94
-0.05	100	12.56	2.158	0.574	20.0	12.95
-0.10	100	12.56	2.158	0.539	20.0	12.96
-0.20	100	12.56	2.158	0.471	20.0	12.96
-0.30	100	12.56	2.158	0.407	20.0	12.97
-0.40	100	12.56	2.158	0.347	20.0	12.98
-0.50	100	12.56	2.158	0.293	20.0	12.98
-0.70	100	12.56	2.158	0.203	20.0	12.99
-0.80	100	12.56	2.158	0.167	20.0	12.99
-0.90	100	12.56	2.158	0.136	20.0	13.00
-1.00	100	12.56	2.158	0.111	20.0	13.00
-1.15	100	12.56	2.158	0.081	20.0	13.00
-1.20	100	12.56	2.158	0.072	20.0	13.00
-1.55	100	12.56	2.158	0.033	20.0	13.01

Table 7. Recipes for the preparation of MORPH-ASP-PVA membranes.

Log (c_{base} / c_{acid})	**Volume H_20 / mL**	**Mass NaOH / g**	**Mass MORPH/ g**	**Mass ASP / g**	**Mass PVA / g**	**Mass GDGE / g per 90 g reaction mixture**
1.1	100	12.56	2.111	2.317	20.0	12.78
1.0	100	12.56	2.111	2.354	20.0	12.78
0.6	100	12.56	2.111	2.592	20.0	12.74
0.5	100	12.56	2.111	2.677	20.0	12.73
0.4	100	12.56	2.111	2.773	20.0	12.71
0.3	100	12.56	2.111	2.879	20.0	12.70
0.0	100	12.56	2.111	3.237	20.0	12.65
-0.1	100	12.56	2.111	3.361	20.0	12.63
-0.3	100	12.56	2.111	3.596	20.0	12.60
-0.4	100	12.56	2.111	3.702	20.0	12.58
-0.7	100	12.56	2.111	3.958	20.0	12.54
-0.8	100	12.56	2.111	4.021	20.0	12.54
-0.9	100	12.56	2.111	4.075	20.0	12.53
-1.0	100	12.56	2.111	4.120	20.0	12.52

Table 8. Recipes for the preparation of TRIS-ASP-PVA membranes.

Log (c_{base} / c_{acid})	**Volume H_20 / mL**	**Mass NaOH / g**	**Mass TRIS / g**	**Mass ASP / g**	**Mass PVA / g**	**Mass GDGE / g per 90 g reaction mixture**
1.1	100	11.35	1.964	0.159	20.0	13.13
1.0	100	11.38	1.964	0.196	20.0	13.12
0.8	100	11.44	1.964	0.295	20.0	13.11
0.7	100	11.47	1.964	0.359	20.0	13.10
0.3	100	11.70	1.964	0.721	20.0	13.04
0.2	100	11.76	1.964	0.835	20.0	13.02
-0.1	100	11.98	1.964	1.203	20.0	12.97
-0.2	100	12.06	1.964	1.323	20.0	12.95
-0.6	100	12.30	1.964	1.725	20.0	12.89
-0.7	100	12.34	1.964	1.799	20.0	12.88
-0.9	100	12.41	1.964	1.917	20.0	12.86
-1.0	100	12.44	1.964	1.962	20.0	12.85

Table 9. Recipes for the preparation of PROP-ASP-PVA membranes.

Log (c_{base} / c_{acid})	**Volume H_20 / mL**	**Mass NaOH / g**	**Mass PROP / g**	**Mass ASP / g**	**Mass PVA / g**	**Mass GDGE / g per 90 g reaction mixture**
1.7	100	11.29	1.218	0.042	20.0	13.22
1.6	100	11.29	1.218	0.053	20.0	13.22
1.1	100	11.35	1.218	0.159	20.0	13.20
1.0	100	11.38	1.218	0.196	20.0	13.20
0.8	100	11.44	1.218	0.295	20.0	13.18
0.7	100	11.48	1.218	0.359	20.0	13.17
0.6	100	11.52	1.218	0.433	20.0	13.16
0.5	100	11.57	1.218	0.519	20.0	13.15
0.2	100	11.76	1.218	0.835	20.0	13.10
0.1	100	11.83	1.218	0.955	20.0	13.08
-0.4	100	12.19	1.218	1.54	20.0	12.99
-0.5	100	12.25	1.218	1.640	20.0	12.97
-0.8	100	12.38	1.218	1.799	20.0	12.94
-0.9	100	12.41	1.218	1.863	20.0	12.93

Table 10. Recipes for the preparation of DEAPA-ASP-PVA membranes.

Log (c_{base} / c_{acid})	**Volume H_20 / mL**	**Mass NaOH / g**	**Mass DEAPA / g**	**Mass ASP / g**	**Mass PVA / g**	**Mass GDGE / g per 90 g reaction mixture**
1.1	100	11.35	2.112	0.159	20.0	13.11
1	100	11.38	2.112	0.196	20.0	13.11
0.6	100	11.52	2.112	0.433	20.0	13.07
0.5	100	11.57	2.112	0.519	20.0	13.06
0.3	100	11.69	2.112	0.721	20.0	13.03
0.2	100	11.76	2.112	0.835	20.0	13.01
-0.2	100	12.06	2.112	1.323	20.0	12.93
-0.3	100	12.12	2.112	1.438	20.0	12.92
-0.5	100	12.25	2.112	1.640	20.0	12.89
-0.6	100	12.29	2.112	1.725	20.0	12.87
-0.7	100	12.34	2.112	1.799	20.0	12.86
-0.8	100	12.38	2.112	1.863	20.0	12.85

[19]. A 1000 V, 1200 mA power supply (E-C Apparatus, Co., Holbrook, NY, USA) was used for all IET experiments. The intermembrane distance was about 1 mm and the active membrane surface area was about 15 cm^2. Joule heat was dissipated by recirculating ice water through the glass jackets of the containers that held the anolyte, anodic separation compartment solution, cathodic separation compartment solution, and catholyte.

In all of the experiments the anodic membranes were the hydrolytically-stable, IDA-PVA membranes prepared in our laboratory [31], and the anolyte contained 30 mM methanesulfonic acid. For the experiments with the MAL-PROP-PVA, ASP-PROP-PVA, and MORPH-ASP-PVA membranes, LYS-PVA was used as the cathodic membrane[32], and the catholyte contained 60 mM NaOH. For the experiments with the TRIS-ASP-PVA, PROP-ASP-PVA, and DEAPA-ASP-PVA membranes, Q-PVA was used as the cathodic membrane [30], and the catholyte contained 180 mM NaOH. The separation membranes were the PVA-based buffering membranes that had tunable pH values synthesized according to Section 3.2. In all cases, the anodic separation compartment initially contained an aqueous solution of 10 mM IDA, and 1 mM of a pI marker. For the experiments with the MAL-PROP-PVA, ASP-PROP-PVA, and MORPH-ASP-PVA membranes, the cathodic separation compartment initially contained an aqueous solution of 10 mM histidine, and 1 mM of the same pI marker that was in the anodic separation compartment. For the experiments with the TRIS-ASP-PVA, PROP-ASP-PVA, and DEAPA-ASP-PVA membranes, the cathodic separation compartment

initially contained an aqueous solution of 10 mM arginine, and 1 mM of the same pI marker that was used in the anodic separation compartment. Table 11 lists the pI markers used, their abbreviation, and pI values. IET separations in the BF200IET unit were carried out in the recirculating mode [19], at feed and collection stream flow rates of 30 mL/min, and anolyte and catholyte flow rates of 2 L/min.

Fractions were collected from all streams at pre-set times and analyzed by CE. Three different CE methods were used, depending on the experiment. The composition and pH of the background electrolyte (BGE) used in the CE analyses, as well as the applied potential, inner diameter (i.d.) of the capillary, total length of the capillary (L_t), and length of the capillary from inlet to detector (L_d) are listed in Table 12. All of the analyses were done at a detector wavelength of 214 nm. One of the pI markers, however, is not UV-absorbing (aspartic acid), therefore an additional step was done before they were analyzed by CE. After the sample containing the non-UV-absorbing marker was collected from the IET experiment, a 200 µL aliquot of it was taken and added to 200 µL of a 25 mM boric acid / LiOH buffer (pH = 10.0), followed by 5 µL of a 40 mM KCN, and 35 µL of a 10 mM naphthalene-2,3-dicarboxaldehyde solution. The mixture was shaken and left at room temperature for 10 minutes prior to analysis by CE. This produced an N-substituted 1-cyanobenzisoindole [61] derivative of the pI marker that was easily detected at 214 nm.

Table 11. The pI markers used in the membrane pH determination experiments, their abbreviation, and pI value.

Compound	Abbreviation	pI
Aspartic acid	MK-2.7	2.7
Dansyl-aspartic acid	MK-3.2	3.2
Nicotinic acid	MK-3.4	3.4
p-Aminobenzoic acid	MK-3.6	3.6
ElphoMark™ 3.9	EM-3.9	3.9
2-Pyridyl acetic acid	MK-4.2	4.2
3-Pyridyl acetic acid	MK-4.3	4.3
4-Pyridyl acetic acid	MK-4.4	4.4
ElphoMark™ 4.6	EM-4.6	4.6
3-Pyridine propionic acid	MK-4.8	4.8
ElphoMark™ 5.2	EM-5.2	5.2
ElphoMark™ 5.6	EM-5.6	5.6
ElphoMark™ 5.9	EM-5.9	5.9
ElphoMark™ 6.2	EM-6.3	6.3
ElphoMark™ 6.7	EM-6.7	6.7
5-Hydroxy-2-methylpyridine	MK-7.2	7.2
Histidine	MK-7.6	7.6
Carnosine	MK-8.1	8.1
ElphoMark™ 8.3	EM-8.3	8.3
ElphoMark™ 8.7	EM-8.7	8.7
ElphoMark™ 9.3	EM-9.3	9.3
ElphoMark™ 9.6	EM-9.6	9.6
ElphoMark™ 10.0	EM-10.0	10

Table 12. CE methods used to analyze fractions from the IET pH determination experiments.

Method	Capillary			BGE					Potential
				Composition				pH	
	I.d. / µm	L_t / cm	L_d / cm	Acid		Base			
				Name	Conc. / mM	Name	Conc. / mM		
1	27	26.5	19.8	Citric acid	25	LiOH	18	3.4	10 kV
2	27	26.5	19.8	Phosphoric acid	25	LiOH	37	6.9	10 kV
3	27	26.5	19.8	Boric acid	20	LiOH	15	9.2	10 kV

Figure 24 shows the instrument set-up for two experiments as examples of how the membranes were tested. The first experiment (Figure 24a) utilized a PROP-ASP-PVA membrane with log (c_{base} / c_{acid}) = 0.2, where c_{base} and c_{acid} are the concentrations of the unprotonated and protonated forms of PROP. In the second experiment (Figure 24b), another PROP-ASP-PVA membrane was used as the separation membrane. The composition of this membrane was such that log (c_{base} / c_{acid}) = 0.1. The pH of the membranes in these two experiments should therefore differ by only 0.1. A solution containing 1 mM of MK-7.6 and a pH-biaser [20], IDA, at a concentration of 10 mM was placed in the anodic separation compartment and a solution containing 1 mM MK-7.6 and a pH-biaser [20], arginine, at a concentration of 10 mM was placed in the cathodic separation compartment in both experiments. If, as shown in Figure 24a, all of MK-7.6 were to migrate into the anodic separation compartment (ASC), the pH of the separation membrane would have to be higher than 7.6. If, in the second experiment (as shown in Figure 24b), all of the MK-7.6 were to migrate into the cathodic separation compartment (CSC), the pH of the membrane with that composition would have to be lower than 7.6. Figures 25 and 26 show the CE traces of the samples taken from the compartments at the beginning and end of each experiment. Clearly, all of MK-7.6 migrated into the ASC in the first experiment, and all of MK-7.6 migrated into the CSC in the second experiment.

Tables 13-18 lists the pI markers used for each experiment, the IET run time, the initial and final IET voltage, the initial and final IET current, the CE method used to analyze

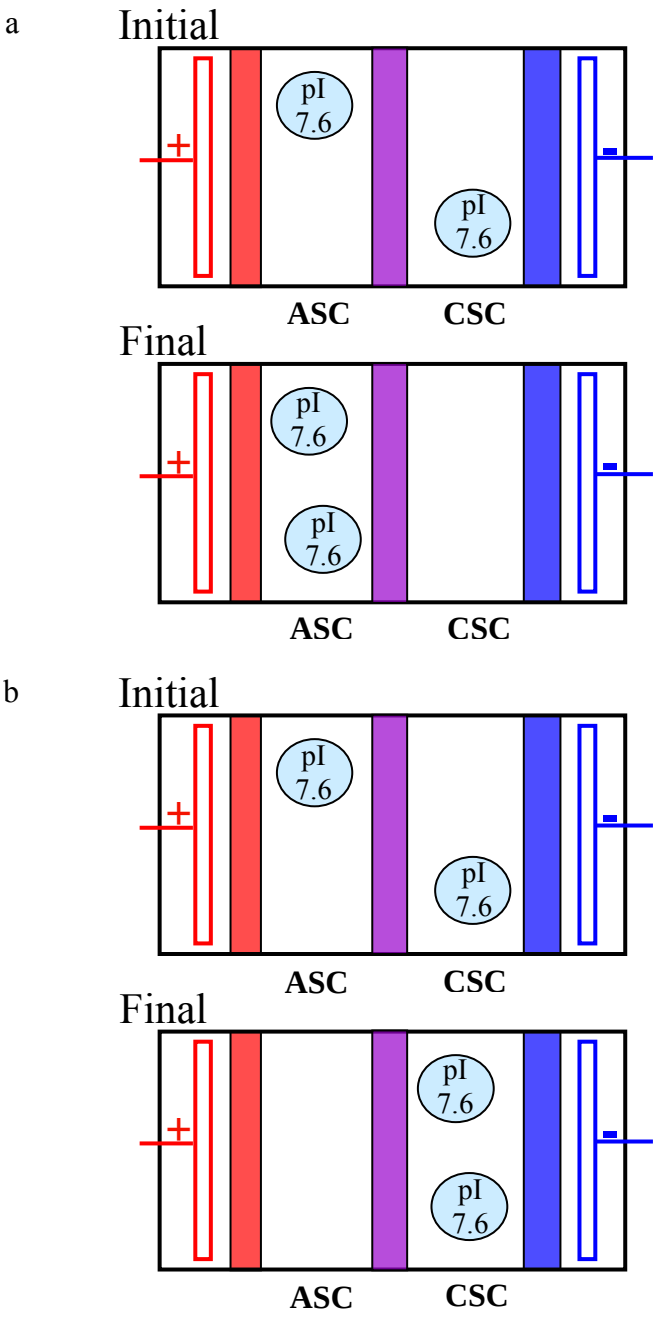


Figure 24. a) Schematic representation of the initial and final conditions of the IET experiment with a PROP-ASP-PVA membrane having a log (c_{base} / c_{acid}) = 0.2 as the separation membrane. b) Schematic representation of the initial and final conditions of the IET experiment with a PROP-ASP-PVA membrane having a log (c_{base} / c_{acid}) = 0.1 as the separation membrane. ASC: anodic separation compartment; CSC: cathodic separation compartment.

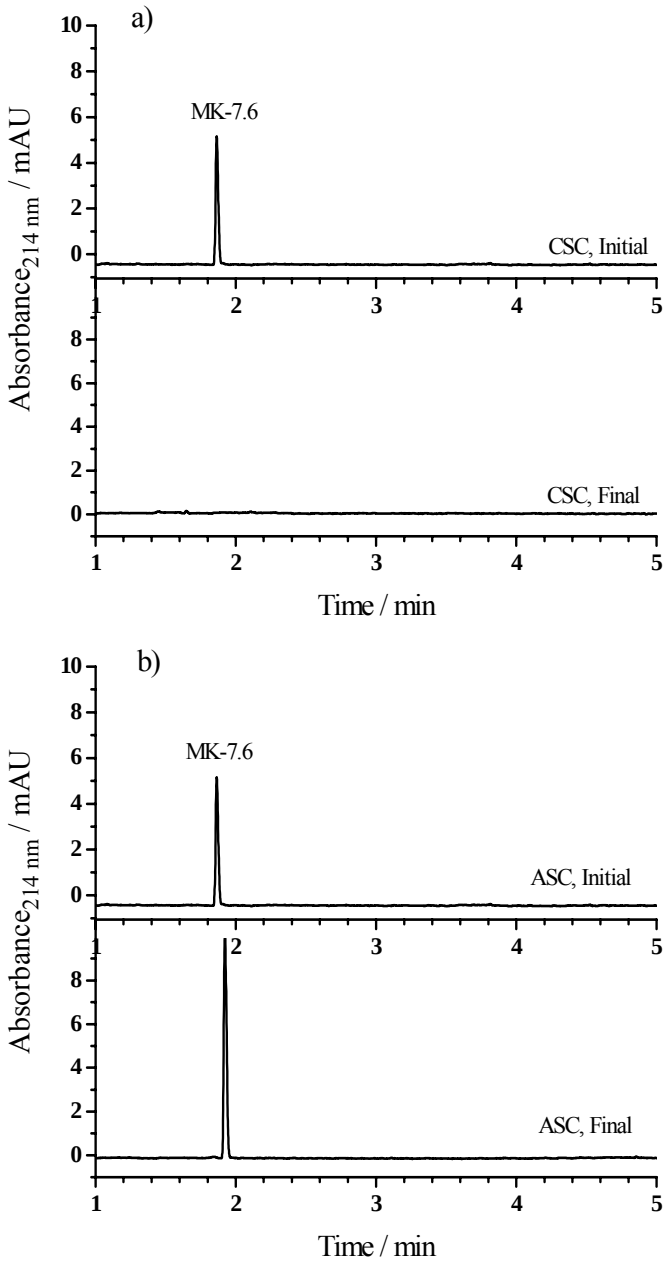


Figure 25. CE traces of the initial and final samples taken from the cathodic separation compartment (CSC) (a), and the anodic separation compartment (ASC) (b) in the membrane pH determination IET experiment using the log (c_{base} / c_{acid}) = 0.2 PROP-ASP-PVA membrane as the separation membrane.

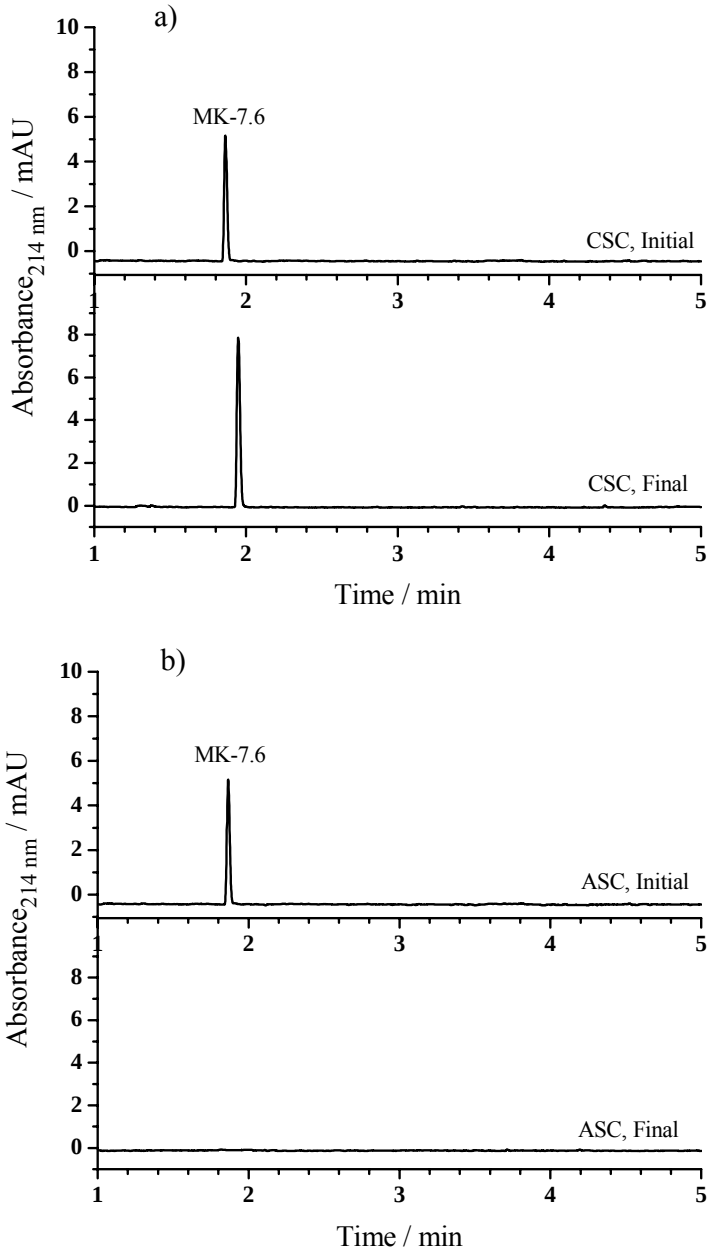


Figure 26. CE traces of the initial and final samples taken from the cathodic separation compartment (CSC) (a), and the anodic separation compartment (ASC) (b) in the membrane pH determination IET experiment using the log (c_{base} / c_{acid}) = 0.1 PROP-ASP-PVA membrane as the separation membrane.

Table 13. IET parameters for experiments with the MAL-PROP-PVA membranes.

Log (c_{base} / c_{acid})	pI marker	IET run time / min	Initial IET voltage / V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE Method	Final IET compartment
1.5	EM-6.3	35	300	400	150	52	1	CSC
1.5	EM-5.9	35	300	400	150	52	1	ASC
1.0	MK-4.8	50	59	400	250	89	2	ASC
0.9	MK-4.8	50	61	400	250	84	2	CSC
0.7	EM-4.6	45	47	400	250	92	2	ASC
0.6	EM-4.6	45	55	400	250	97	2	CSC
0.6	MK-4.4	50	49	400	250	83	2	ASC
0.5	MK-4.4	50	64	400	300	79	2	CSC
0.4	MK-4.2	50	72	400	300	62	1	ASC
0.3	MK-4.2	50	70	350	300	59	1	CSC
0.2	EM-3.9	40	68	350	300	62	1	ASC
0.1	EM-3.9	45	64	350	300	71	1	CSC
0.0	MK-3.6	40	70	350	300	65	1	ASC
-0.1	MK-3.6	50	72	350	300	70	1	CSC
-0.4	MK-3.4	50	67	350	300	82	1	ASC
-0.5	MK-3.4	55	73	350	300	76	1	CSC
-0.6	MK-3.2	45	49	400	250	92	3	ASC
-0.7	MK-3.2	45	51	400	250	99	3	CSC
-0.9	MK-2.7	45	58	400	250	87	3	ASC
-1.0	MK-2.7	45	42	400	250	94	3	CSC

Table 14. IET parameters for the experiments with the ASP-PROP-PVA buffering membranes

Log (c_{base} / c_{acid})	pI marker	IET run time / min	Initial IET voltage / V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE Method	Final IET compartment
1.30	EM-6.3	60	76	300	200	41	2	ASC
1.20	EM-6.3	60	59	250	200	52	2	CSC
0.95	EM-5.9	65	60	325	200	61	1	ASC
0.80	EM-5.9	70	48	250	150	33	1	CSC
0.60	EM-5.2	55	100	350	167	101	2	ASC
0.50	EM-5.2	55	97	300	150	82	2	CSC
0.25	MK-4.8	65	100	250	172	87	1	ASC
0.20	MK-4.8	50	150	250	150	118	1	CSC
-0.05	EM-4.6	60	250	250	148	57	1	ASC
-0.10	EM-4.6	60	250	250	150	46	1	CSC
-0.20	MK-4.4	60	200	300	173	51	1	ASC
-0.30	MK-4.4	60	200	300	160	62	1	CSC
-0.30	MK-4.3	60	101	250	150	47	1	ASC
-0.40	MK-4.3	60	124	250	150	61	1	CSC
-0.40	MK-4.2	55	112	250	150	51	1	ASC
-0.50	MK-4.3	55	119	250	150	59	1	CSC
-0.70	EM-3.9	60	99	300	150	94	2	ASC
-0.80	EM-3.9	60	127	325	150	105	2	CSC
-0.90	MK-3.6	60	190	250	150	63	1	ASC
-1.00	MK-3.6	60	75	250	300	69	1	CSC
-1.15	MK-3.4	55	89	250	300	74	1	ASC
-1.20	MK-3.4	55	96	250	300	81	1	CSC
-1.55	MK-3.2	40	82	250	300	79	1	CSC
-1.55	MK-2.7	40	82	250	300	79	3	ASC

Table 15. IET parameters for the experiments with the MORPH-ASP-PVA buffering membranes

Log (c_{base} / c_{acid})	pI marker	IET run time / min	Initial IET voltage / V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE Method	IET final compartment
1.1	EM-6.7	60	300	400	179	47	2	ASC
1.0	EM-6.7	60	300	400	142	55	2	CSC
0.6	EM-6.3	60	250	300	126	62	2	ASC
0.5	EM-6.3	60	250	350	153	49	2	CSC
0.4	EM-5.9	55	149	300	250	72	2	ASC
0.3	EM-5.9	55	176	300	250	91	2	CSC
0.0	EM-5.6	60	199	350	250	100	2	ASC
-0.1	EM-5.6	60	200	350	194	87	2	CSC
-0.3	EM-5.2	60	225	300	197	68	2	ASC
-0.4	EM-5.2	60	225	300	184	74	2	CSC
-0.7	MK-4.8	65	250	300	173	88	2	ASC
-0.8	MK-4.8	65	250	300	201	92	2	CSC
-0.9	EM-4.6	60	200	350	149	94	2	ASC
-1.0	EM-4.6	60	200	350	152	69	2	CSC

Table 16. IET parameters for the experiments with the TRIS-ASP-PVA buffering membranes

Log (c_{base} / c_{acid})	**pI marker**	**IET run time / min**	**Initial IET voltage / V**	**Final IET voltage / V**	**Initial IET current / mA**	**Final IET current / mA**	**CE Method**	**IET final compartment**
1.1	MK-7.6	50	300	500	159	39	2	ASC
1.0	MK-7.6	50	300	500	172	47	2	CSC
0.8	MK-7.2	45	300	500	181	51	2	ASC
0.7	MK-7.2	45	300	500	190	55	2	CSC
0.3	EM-6.7	50	300	450	127	44	2	ASC
0.2	EM-6.7	40	300	450	151	60	2	CSC
-0.1	EM-6.3	50	146	250	250	87	2	ASC
-0.2	EM-6.3	55	167	300	250	81	2	CSC
-0.6	EM-5.9	60	162	300	300	72	2	ASC
-0.7	EM-5.9	60	182	300	300	92	2	CSC
-0.9	EM-5.6	55	137	300	250	79	2	ASC
-1.0	EM-5.6	55	146	300	250	61	2	CSC

Table 17. IET parameters for the experiments with the PROP-ASP-PVA buffering membranes.

Log (c_{base} / c_{acid})	pI marker	IET run time / min	Initial IET voltage / V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE Method	IET final compartment
1.7	EM-9.3	55	100	150	257	150	3	ASC
1.6	EM-9.3	60	94	159	300	150	3	CSC
1.1	EM-8.7	60	88	179	300	150	3	ASC
1.0	EM-8.7	60	107	182	300	150	3	CSC
0.8	EM-8.3	65	79	187	300	149	2	ASC
0.7	EM-8.3	65	89	200	300	146	2	CSC
0.6	MK-8.1	60	110	200	300	150	2	ASC
0.5	MK-8.1	60	98	200	300	124	2	CSC
0.2	MK-7.6	55	72	250	250	97	2	ASC
0.1	MK-7.6	55	67	250	250	84	2	CSC
-0.4	MK-7.2	50	73	300	250	74	2	ASC
-0.5	MK-7.2	50	80	300	250	69	2	CSC
-0.8	EM-6.7	60	87	300	300	81	2	ASC
-0.9	EM-6.7	60	100	300	300	79	2	CSC

Table 18. IET parameters for the experiments with the DEAPA-ASP-PVA buffering membranes.

Log (c_{base} / c_{acid})	pI marker	IET run time / min	Initial IET voltage / V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE method	IET final compartment
1.1	EM-10.0	50	47	69	250	250	3	ASC
1.0	EM-10.0	55	52	64	250	250	3	CSC
0.6	EM-9.6	60	51	89	250	250	3	ASC
0.5	EM-9.6	55	59	77	250	250	3	CSC
0.3	EM-9.3	65	61	93	250	250	3	ASC
0.2	EM-9.3	65	67	92	250	250	3	CSC
-0.2	EM-8.7	60	60	87	250	250	2	ASC
-0.3	EM-8.7	55	59	92	250	250	2	CSC
-0.5	EM-8.3	60	64	100	300	157	2	ASC
-0.6	EM-8.3	60	59	100	300	162	2	CSC
-0.7	MK-8.1	60	67	100	300	152	2	ASC
-0.8	MK-8.1	65	75	100	300	124	2	CSC

the samples taken from the sample compartments, and the compartment in which the pI marker accumulated at the end of the run (either the anodic separation compartment ASC, or the cathodic separation compartment, CSC) for the experiments with the MAL-PROP-PVA, ASP-PROP-PVA, MORPH-PROP-PVA, TRIS-PROP-PVA, PROP-ASP-PVA, and DEAPA-ASP-PVA membranes, respectively.

3.3.1 Final results and conclusions for the determination of the membrane pH values

A graph of the results of the IET experiments with the membranes whose pH is tunable is shown in Figure 27. The x-axis represents the log (c_{base} / c_{acid}) values corresponding to a particular membrane composition. The membrane cannot have a pH equal to, or higher than, the value shown on the left (red) axis. Also, the membrane cannot have a pH equal to, or lower than, the value shown on the right (blue) axis.

For all series of membranes, the pK_a values of the incorporated buffering compound differ slightly from the pK_a values of the free compounds. This is likely due to the fact that the amino groups of the buffering compounds are substituted with 2-hydroxypropyl groups from GDGE, and/or they are in close proximity to the secondary alcohol groups of PVA. As can be seen, the membranes cover the $3 < pH < 10$ range, and at a corresponding membrane composition (log (c_{base} / c_{acid}) value), each series is no more than 1.5 pH units apart from the next. This insures good buffering capacity across the entire pH range. The availability of membranes that have $\left| \log (c_{base} / c_{acid}) \right| < 0.75$

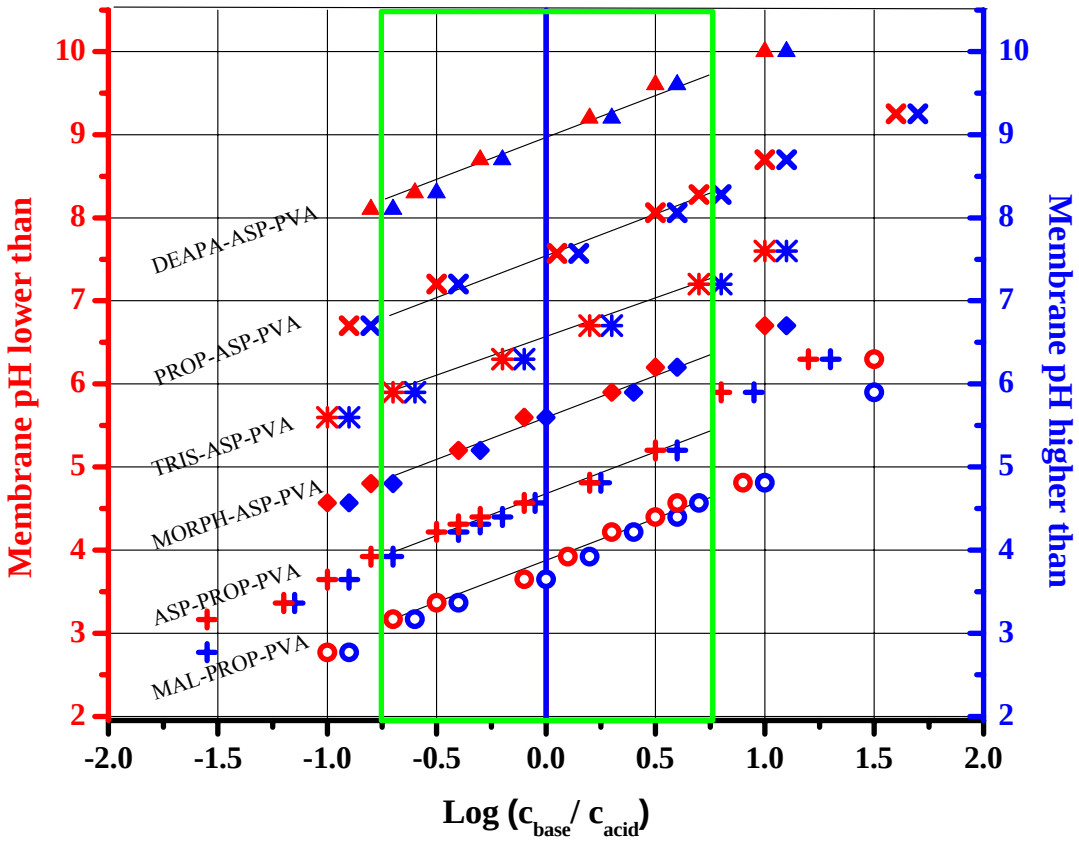


Figure 27. Graph of membrane pH versus composition expressed as log (c_{base} / c_{acid}). Lines represent (from top): DEAPA-ASP-PVA membranes; PROP-ASP-PVA membranes, TRIS-ASP-PVA membranes, MORPH-ASP-PVA membranes, ASP-PROP-PVA membranes, MAL-PROP-PVA membranes.

(shown by the green box in Figure 27) allows for membranes to be chosen that have the best buffering capacity.

For the DEAPA-ASP-PVA, PROP-ASP-PVA, TRIS-ASP-PVA, and MORPH-ASP-PVA membranes, the points plotted on the graph line up well with straight lines of unit slopes (the theoretical slope value according to the Henderson – Hasselbach equation) that can be drawn through them. For the ASP-PROP-PVA membranes, however, linearity is lost at membrane compositions with log (c_{base} / c_{acid}) > 0.75. For this pH range, the MORPH-ASP-PVA membrane series can be used to ensure accurate pH values.

For each series of membranes, the points in Figure 27 in the $\left| \log (c_{base} / c_{acid}) \right| < 0.75$ range were analyzed using the linear regression feature of Origin 7.5. The slope was set at 1, since this is the theoretical slope according to the Henderson-Hasselbach equation. The pH value of the membrane at x = 0 yields the apparent pK_a of the functional group in the membrane that is providing the buffering. The results of the linear curve fits are listed in Table 19, along with the R^2 values for the line and the standard deviation (SD). All of the R^2 values for the fits are > 0.98, indicating the regression fits well with the experimental data points.

Table 19. List of the apparent pK_a values for the tunable PVA-based buffering membranes obtained from the linear fits of the points in Figure 27 using Origin 7.5.

Membrane	Apparent pK_a	SD	R^2
MAL-PROP-PVA	3.82	0.09	0.987
ASP-PROP-PVA	4.65	0.05	0.992
MORPH-ASP-PVA	5.59	0.07	0.991
TRIS-ASP-PVA	6.48	0.07	0.994
PROP-ASP-PVA	7.54	0.09	0.987
DEAPA-ASP-PVA	8.93	0.09	0.994

3.3.2 Additional experiments with tunable pH membranes

3.3.2.1 Membranes buffering on the ASP amino group

ASP, when used as a titrant, adds an additional amino group to each of the membrane mixtures. Since this amino group could potentially be playing a role in the buffering of the mid and high pH membrane series, membranes were synthesized to determine the pK_a of the conjugate acid of the amino group in ASP. This requires a compound to titrate the amino group of ASP that is much more basic than the amino group of ASP. One such compound, 1,3,4,6,7,8-hexahydro-2*H*-pyrimido[1,2-*a*]pyrimidine, or PYR, has a very high pK_a of around 13 [53] for its conjugate acid form. Even a hydroxyalkyl-substituted form of this compound has a pK_a of around 13 for the conjugate acid form [53]. This compound, therefore, would be expected to remain a strongly basic titrating

group even when incorporated into the membrane matrix, and was therefore chosen as a titrant for membranes buffering on the amino group of ASP.

Three membranes were synthesized using ASP and PYR. The recipes for these three membranes are listed in Table 20. The amounts listed are for a PVA paper substrate with dimensions of 24 cm by 19 cm. The membranes were tested as separation membranes in an IET experiment using a modified BF200IET [19]. In all experiments, the anodic membrane was an IDA-PVA membrane, and the cathodic membrane was a Q-PVA membrane. The anolyte contained 30 mM methanesulfonic acid, and the catholyte contained 180 mM NaOH. The ASC initially contained a mixture of two pI markers, each at a concentration of 1 mM, and 10 mM IDA as the pH-biaser [20]. The CSC initially contained a mixture of two pI markers, each at a concentration of 1 mM, and 10 mM arginine as the pH biaser [20]. Table 21 lists the membrane compositions tested, the pI markers used for each experiment, the IET run time, the initial and final IET voltage, the initial and final IET current, the CE methods used to analyze the fractions taken from the sample compartments, and the final IET compartment in which the pI marker accumulated.

The results of the experiments are graphed in Figure 28. The x-axis on the graph represents the log (c_{base} / c_{acid}) values for the respective membrane compositions. As in Figure 27, the membrane cannot have a pH equal to, or higher than, the value shown on

Table 20. Recipes for the preparation of membranes buffering on the less basic amino group in the ASP-PYR mixture.

Log (c_{base} / c_{acid})	Volume H_20 / mL	Mass NaOH / g	Mass ASP / g	Mass PYR / g	Mass PVA / g	Mass GDGE / g per 25 g reaction mixture
0.5	25	3.25	0.532	1.531	5.0	3.62
0.0	25	3.25	0.532	1.392	5.0	3.62
-0.5	25	3.25	0.532	1.253	5.0	3.62

Table 21. IET parameters for the experiments with membranes buffering on the less basic amino group in the ASP-PYR mixture.

Log (c_{base} / c_{acid})	pI marker	IET run time / min	Initial IET voltage / V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE Method	IET final compart.
0.5	MK-10.3	30	244	300	250	130	2	CSC
	EM-10.0							ACS
0.0	EM-10.0	30	250	300	190	94	2	CSC
	EM-9.6							ASC
-0.5	EM-9.6	30	210	250	250	98	2	CSC
	EM-9.3							ASC

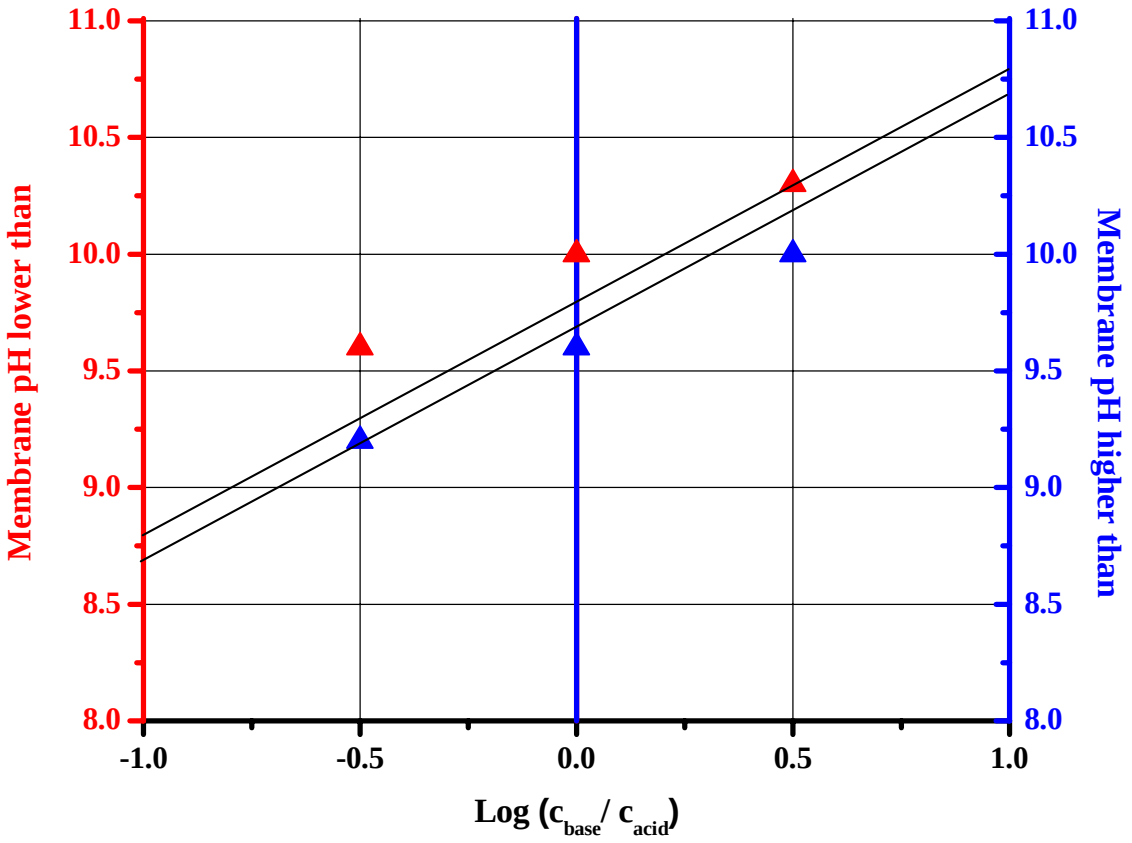


Figure 28. Graph of membrane pH versus composition, expressed as log (c_{base} / c_{acid}), for the membranes that buffer on the less basic amino group in the ASP-PYR mixture.

the left (red) axis. Also, the membrane cannot have a pH equal to, or lower than, the value shown on the right (blue) axis. The pH values of the membranes are in the range that is delimited by the upper and lower boundaries set by the pI markers (shown as black diagonal lines in Figure 28). The pK_a of the conjugate acid form of the amino group in ASP in the membrane is in the 9.65 to 9.75 range based on the upper and lower boundaries set by the pI markers in Figure 28. This amino group is clearly more basic than the buffering amino groups in the MORPH-ASP-PVA, TRIS-ASP-PVA, PROP-ASP-PVA, and DEAPA-PVA membranes. This membrane series allows us to extend the pH range of the membranes to higher values while staying within the $-0.75 < \log (c_{base} / c_{acid}) < 0.75$ range.

3.3.2.2 Membranes buffering on the other amino group in the MORPH-ASP and DEAPA-ASP mixtures

Since MORPH and DEAPA are diamines, additional experiments were done to test the pH of MORPH-ASP-PVA membranes that buffered on the more basic amino group in the MORPH-ASP mixture, and the DEAPA-ASP-PVA membranes that buffered on the least basic amino group in the DEAPA-ASP mixture. The membranes were prepared in the same way as the membranes described in Section 3.2. Tables 22 and 23 show the volume of water, and amounts of NaOH, MORPH (or DEAPA), ASP, PVA and GDGE for the given membrane compositions. The membranes were tested as separation membranes in an IET experiment using a modified BF200IET [19]. In all experiments, the anodic membrane was an IDA-PVA membrane, and the cathodic membrane was a

Q-PVA membrane. The anolyte contained 30 mM methanesulfonic acid, and the catholyte contained 180 mM NaOH. The ASC initially contained a mixture of two pI markers, each at a concentration of 1 mM, and 10 mM IDA as the pH-biaser[20]. The CSC initially contained a mixture of two pI markers, each at a concentration of 1 mM, and 10 mM arginine as the pH biaser [20]. Tables 24 and 25 list the membrane compositions tested, the pI markers used for each experiment, the IET run time, the initial and final IET voltage, the initial and final IET current, the CE methods used to analyze the fractions taken from the sample compartments, and the final IET compartment in which the pI marker accumulated.

Table 22. Recipes for the preparation of MORPH-ASP-PVA membranes buffering on the more basic amino group in the MORPH-ASP mixture.

Log (c_{base} / c_{acid})	**Volume H_20 / mL**	**Mass NaOH / g**	**Mass MORPH / g**	**Mass ASP / g**	**Mass PVA / g**	**Mass GDGE / g per 90 g reaction mixture**
1.0	100	11.39	2.111	0.196	20.0	13.11
0.4	100	11.63	2.111	0.615	20.0	12.97
0.0	100	11.91	2.111	1.079	20.0	12.97
-0.7	100	12.34	2.111	1.799	20.0	12.97
-1.0	100	12.44	2.111	1.962	20.0	12.84

Table 23. Recipes for the preparation of DEAPA-ASP-PVA membranes buffering on the least basic amino group in the DEAPA-ASP mixture.

Log (c_{base} / c_{acid})	Volume H_20 / mL	Mass NaOH / g	Mass DEAPA / g	Mass ASP / g	Mass PVA / g	Mass GDGE / g per 90 g reaction mixture
1.0	100	12.56	2.112	2.354	20.0	12.78
0.0	100	12.56	2.112	3.237	20.0	12.65
-1.0	100	12.56	2.112	4.120	20.0	12.52

Table 24. IET parameters for the experiments with MORPH-ASP-PVA membranes that buffer on the more basic amino group in the MORPH-ASP mixture.

Log (c_{base} / c_{acid})	pI marker	IET run time / min	Initial IET voltage / V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE Method	IET final compart.
1.0	EM-9.3	30	202	350	200	67	2	CSC
	EM-8.7							ACS
0.4	MK-8.3	30	300	300	247	81	2	CSC
	MK-8.1							ASC
0.0	MK-8.1	30	196	400	250	73	2	CSC
	MK-7.6							ASC
-0.7	MK-7.2	30	250	400	239	92	2	CSC
	EM-6.7							ASC
-1.0	MK-7.2	30	174	400	250	59	2	CSC
	EM-6.7							ASC

Table 25. IET parameters for the experiments with DEAPA-ASP-PVA membranes that buffer on the least basic amino group in the DEAPA-ASP mixture.

Log (c_{base} / c_{acid})	pI marker	IET run time / min	Initial IET voltage / V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE Method	IET final compart.
1.0	EM-8.7	30	190	350	200	78	2	CSC
	EM-8.3							ACS
0.0	MK-8.1	30	175	350	200	58	2	CSC
	MK-7.6							ASC
-1.0	EM-6.7	30	169	350	200	64	2	CSC
	EM-6.3							ASC

The results of the experiments are graphed in Figures 29 and 30 for the MORPH-ASP-PVA and DEAPA-ASP-PVA membranes, respectively. The x-axis on both graphs represents the log (c_{base} / c_{acid}) values for the respective membrane compositions. As in Figure 27, the membrane cannot have a pH equal to, or higher than, the value shown on the left (red) axis. Also, the membrane cannot have a pH equal to, or lower than, the value shown on the right (blue) axis. The pH values of the membranes are in the range that is delimited by the upper and lower boundaries set by the pI markers (shown as black diagonal lines in Figures 29 and 30). The series that buffers on the more basic amino group in the MORPH-ASP mixture has pH values that are about 2 units higher than the pH values of the membrane series that buffers on the less basic amino group of the MORPH-ASP mixture, as seen in Figure 27. With the DEAPA-ASP-PVA

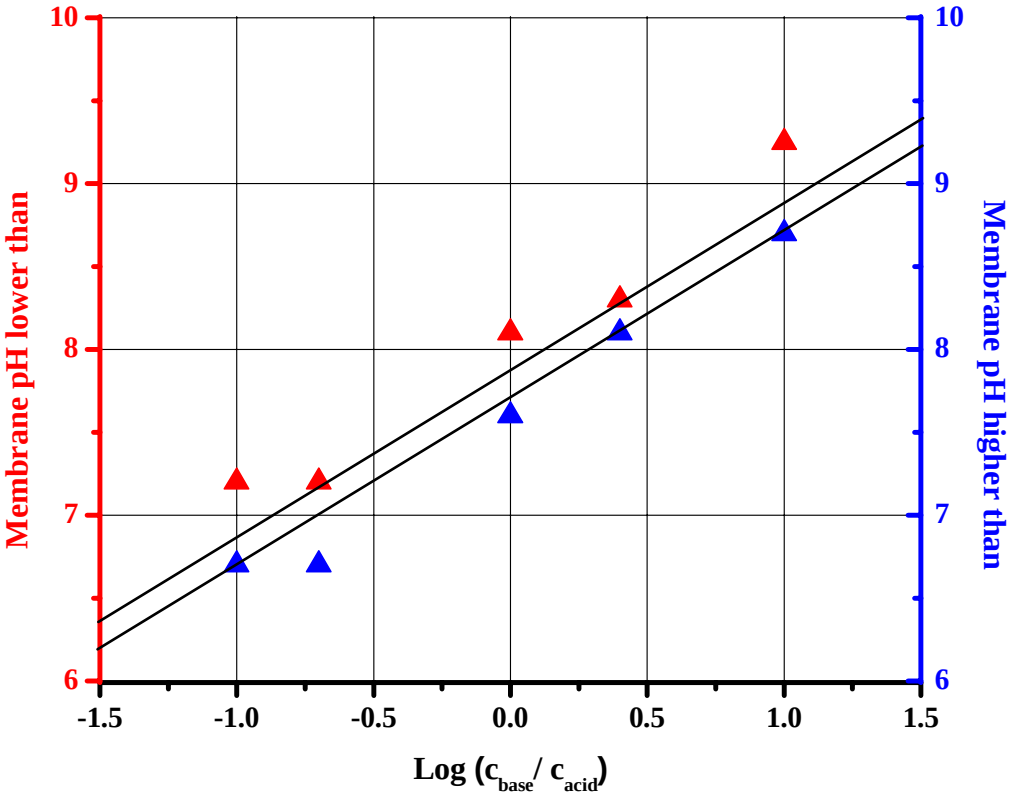


Figure 29. Graph of membrane pH versus composition, expressed as log (c_{base} / c_{acid}), for the MORPH-ASP-PVA membranes that buffer on the more basic amino group in the MORPH-ASP mixture.

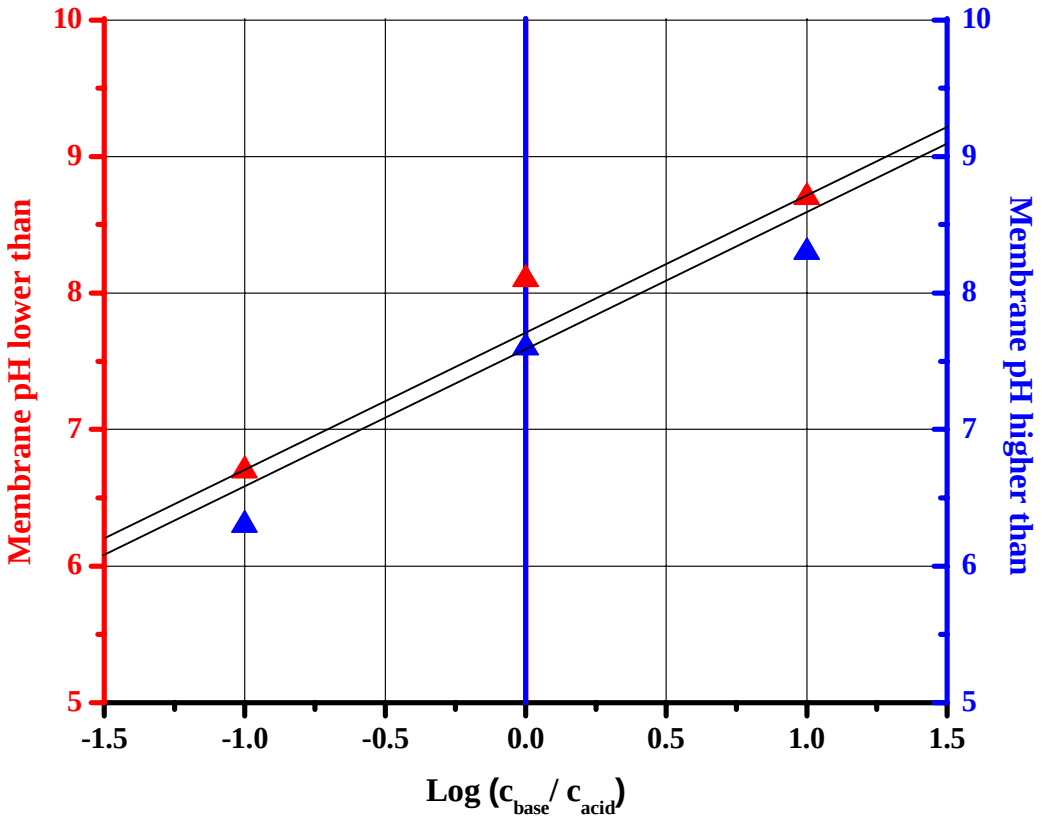


Figure 30. Graph of membrane pH versus composition, expressed as log (c_{base} / c_{acid}), for the DEAPA-ASP-PVA membranes that buffer on the least basic amino group in the DEAPA-ASP mixture.

membranes that buffer on the least basic amino group in the DEAPA-ASP mixture, the membrane pH values are about 2.4 pH units lower than in the membrane series that buffers on the more basic amino group in the DEAPA-ASP mixture, as shown in Figure 27.

3.4 Reproducibility of the tunable PVA membrane preparation procedures

3.4.1 Batch to batch reproducibility

The reproducibility of the membrane preparation procedures was tested by repeating selected experiments described in Section 3.3 with different membrane batches. For all of the membrane series, at least one pair of membranes was tested from three parallel batches (two in addition to the experiments from Section 3.3). Table 26 lists the membrane composition, and the pI marker used for the reproducibility experiments. The additional batches of membranes were tested as separation membranes in the same way as described in Section 3.3. Tables 27-32 list the membrane compositions, IET run time, initial and final IET voltage, initial and final IET current, CE method, and the compartment in which the pI marker accumulated for the experiments with MAL-PROP-PVA, ASP-PROP-PVA, MORPH-ASP-PVA, TRIS-ASP-PVA, PROP-ASP-PVA, and DEAPA-ASP-PVA membranes, respectively.

Table 26. Membranes tested for reproducibility, their compositions, and the pI markers used in the IET experiments.

Membrane	**log (c_{base} / c_{acid})**	**pI marker**
MAL-PROP-PVA	-0.5	MK-3.4
	-0.4	
ASP-PROP-PVA	-0.3	MK-4.4
	-0.2	
MORPH-ASP-PVA	-0.1	EM-5.6
	0.0	
TRIS-ASP-PVA	0.2	EM-6.7
	0.3	
PROP-ASP-PVA	-0.9	EM-6.7
	-0.8	
	0.1	MK-7.6
	0.2	
	1.0	EM-8.7
	1.1	
DEAPA-ASP-PVA	0.2	EM-9.3
	0.3	

Table 27. IET parameters for the reproducibility experiments with the MAL-PROP-PVA membranes.

Batch	Log (c_{base} / c_{acid})	IET run time / min	Initial IET voltage/ V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE method	IET final compart.
1	-0.4	50	67	350	300	82	1	ASC
	-0.5	55	73	350	300	76	1	CSC
2	-0.4	45	82	400	250	54	1	ASC
	-0.5	45	77	400	250	67	1	CSC
3	-0.4	50	91	400	250	49	1	CSC
	-0.5	50	83	400	250	62	1	CSC

Table 28. IET parameters for the reproducibility experiments with the ASP-PROP-PVA membranes.

Batch	Log (c_{base} / c_{acid})	IET run time / min	Initial IET voltage/ V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE method	IET final compart.
1	-0.2	60	200	300	173	51	1	ASC
	-0.3	60	200	300	160	62	1	CSC
2	-0.2	50	300	300	194	47	1	CSC
	-0.3	50	300	300	168	63	1	CSC
3	-0.2	45	300	350	183	71	1	ASC
	-0.3	50	300	350	152	48	1	CSC

Table 29. IET parameters for the reproducibility experiments with the MORPH-ASP-PVA membranes.

Batch	Log (c_{base} / c_{acid})	IET run time / min	Initial IET voltage/ V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE method	IET final compart.
1	0.0	60	199	350	250	100	2	ASC
	-0.1	60	200	350	194	87	2	CSC
2	0.0	50	300	300	202	57	2	ASC
	-0.1	50	300	300	193	69	2	CSC
3	0.0	50	300	300	176	55	2	ASC
	-0.1	50	300	300	184	72	2	CSC

Table 30. IET parameters for the reproducibility experiments with the TRIS-ASP-PVA membranes.

Batch	Log (c_{base} / c_{acid})	IET run time / min	Initial IET voltage/ V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE method	IET final compart.
1	0.3	50	300	450	127	44	2	ASC
	0.2	40	300	450	151	60	2	CSC
2	0.3	45	350	400	157	49	2	ASC
	0.2	45	350	450	146	71	2	CSC
3	0.3	45	350	450	138	68	2	ASC
	0.2	45	350	450	129	81	2	CSC

Table 31. IET parameters for the reproducibility experiments with the PROP-ASP-PVA membranes.

Batch	Log (c_{base} / c_{acid})	IET run time / min	Initial IET voltage/ V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE method	IET final compart.
1	1.1	60	88	179	300	150	3	ASC
	1.0	60	107	182	300	150	3	CSC
	0.2	55	72	250	250	97	2	ASC
	0.1	55	67	250	250	84	2	CSC
	-0.8	60	87	300	300	81	2	ASC
	-0.9	60	100	300	300	79	2	CSC
2	1.1	50	100	200	227	81	2	ASC
	1.0	50	100	200	202	76	2	CSC
	0.2	50	100	300	197	68	2	ASC
	0.1	50	100	300	186	61	2	CSC
	-0.8	55	150	350	237	92	2	ASC
	1.1	55	150	350	219	90	2	CSC
3	1.0	55	100	250	169	73	2	ASC
	0.2	55	100	250	185	85	2	ASC
	0.1	50	150	350	200	99	2	ASC
	-0.8	50	150	350	211	86	2	CSC
	-0.9	50	150	350	199	103	2	ASC
	1.1	50	150	350	187	93	2	CSC

Table 32. IET parameters for the reproducibility experiments with the DEAPA-ASP-PVA membranes.

Batch	Log (c_{base} / c_{acid})	IET run time / min	Initial IET voltage/ V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE method	IET final compart.
1	0.3	65	61	93	250	250	3	ASC
	0.2	65	67	92	250	250	3	CSC
2	0.3	60	89	117	250	250	3	ASC
	0.2	60	72	124	250	250	3	CSC
3	0.3	60	69	142	250	250	3	ASC
	0.2	60	75	127	250	250	3	CSC

A graphical summary of the results are shown in Figures 31 for the MAL-PROP-PVA, ASP-PROP-PVA, MORPH-ASP-PVA, TRIS-ASP-PVA, and DEAPA-ASP-PVA membranes. Figure 32 is a graph of the results for the PROP-ASP-PVA membranes. The results for the PROP-ASP-PVA membranes were graphed separately, because the membrane pairs were tested at three different membrane compositions, yielding values which could not be clearly seen when graphed in Figure 31. Each pair of membranes was selected so that the membrane pH values would bracket a single pI marker. The x-axis on both graphs represents the log (c_{base} / c_{acid}) values corresponding to the different membrane compositions. The z-axis represents the batch number. As in Figure 27, the membrane cannot have a pH equal to, or higher than, the value shown on the left (red) axis. Also, the membrane cannot have a pH equal to, or lower than, the value shown on

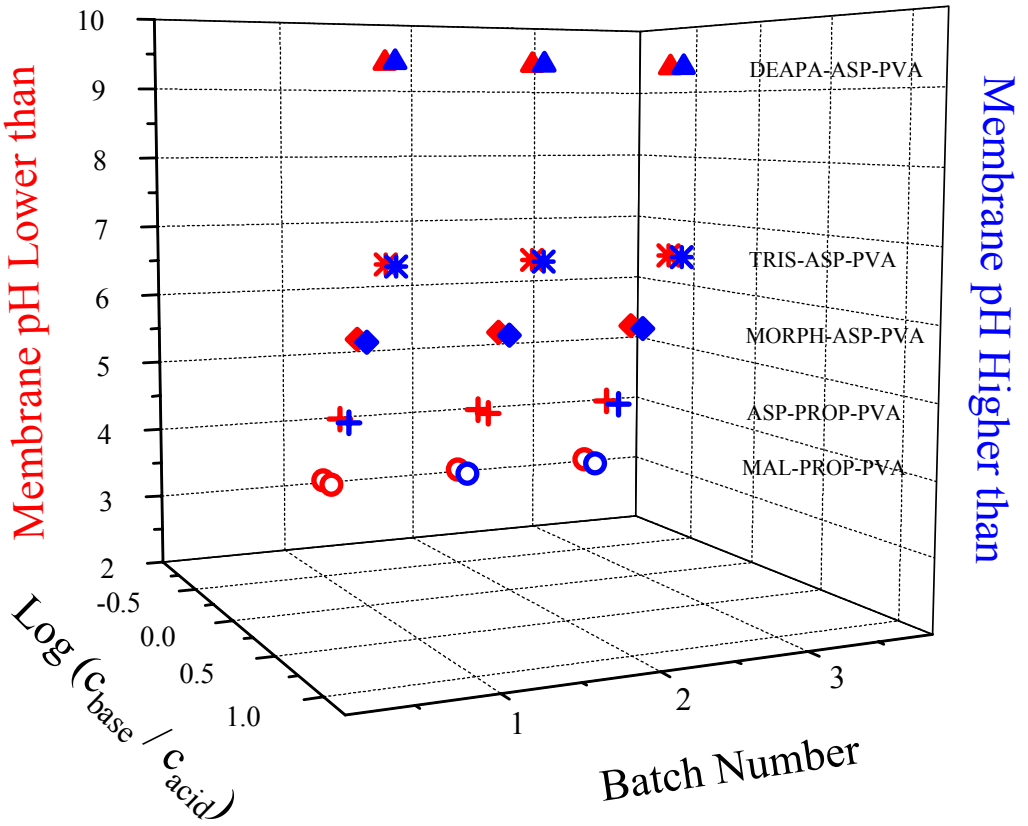


Figure 31. Graph of membrane pH versus composition, expressed as log (c_{base} / c_{acid}) for the three different batches in the reproducibility experiments with MAL-PROP-PVA (symbol circle), ASP-PROP-PVA (symbol cross), MORPH-ASP-PVA (symbol diamond), TRIS-ASP-PVA (symbol asterisk), and DEAPA-ASP-PVA (symbol triangle) membranes.

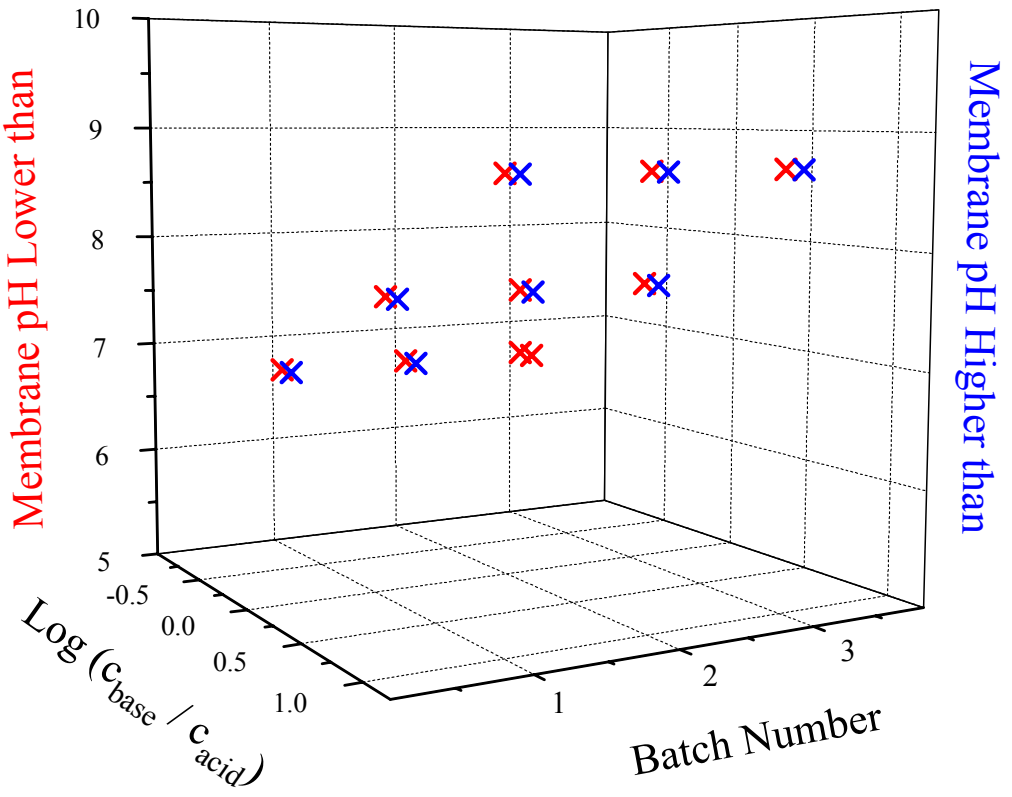


Figure 32. Graph of membrane pH versus composition, expressed as log (c_{base} / c_{acid}) for the three different batches in the reproducibility experiments with the PROP-ASP-PVA membranes.

the right (blue) axis. For membrane pairs that behave as predicted, there should be one red symbol and one blue symbol next to each other. For the experiments with the MORPH-ASP-PVA, TRIS-ASP-PVA, and DEAPA-ASP-PVA membranes, the results from the reproducibility experiments show that the pI marker tested accumulated in the same sample compartment at the end of the run for all three batches tested. With MAL-PROP-PVA, ASP-PROP-PVA, and PROP-ASP-PVA, the results matched for 2 out the three batches. For these batches, at least one of the membrane compositions has a point on the pH value graph (Figure 27) that lies very close to the straight line drawn through the points. This means that the membrane pH is very close the pI of the marker, therefore, a slight difference in weighing of the buffering and titrating species when synthesizing the membranes, or a slight difference in temperature during the IET process could result in the pI marker accumulating in the other separation compartment. Of the 24 membrane pairs that were tested, 21 pairs directed the pI markers into the expected separation compartments, indicating that the tunable PVA-based membranes can be produced reproducibly.

3.4.2 Person to person reproducibility

Experiments were also done to determine if the membranes could be reproduced not only between batches, but between experimenters as well. Two graduate students in our research laboratory were asked to each make three batches of a pair of membranes. The membranes synthesized were the TRIS-ASP-PVA membranes, with compositions of log (c_{base} / c_{acid}) equal to 0.2 and 0.3. These membranes were tested in the same way as

described in Section 3.4.1, using MK-6.7 as the test pI marker. The IET run time, initial and final IET voltage, initial and final IET current, CE method, and the compartment in which the pI marker accumulated for these experiments are listed in Tables 33 and 34.

The results for these experiments are graphed in Figures 33 and 34 for each experimenter. The x-axis on both graphs represents the log (c_{base} / c_{acid}) values corresponding to the different membrane compositions. The z-axis represents the batch number of the membranes synthesized by a particular experimenter. As in Figure 27, the membrane cannot have a pH equal to, or higher than, the value shown on the left (red) axis. Also, the membrane cannot have a pH equal to, or lower than, the value shown on the right (blue) axis. For membrane pairs that behave as predicted, there should be one red symbol and one blue symbol next to each other. From the results, it can be seen that five out of the six membrane batches directed the pI marker into the expected separation compartment.

Table 33. IET parameters for the reproducibility experiments with the TRIS-ASP-PVA membranes synthesized by Experimenter 1.

Batch	Log (c_{base} / c_{acid})	IET run time / min	Initial IET voltage/ V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE method	IET final compart.
1	0.3	50	338	400	200	97	2	ASC
	0.2	55	500	411	101	150	2	CSC
2	0.3	55	450	450	227	107	2	ASC
	0.2	55	400	450	248	99	2	CSC
3	0.3	50	450	475	237	103	2	ASC
	0.2	55	432	450	250	87	2	CSC

Table 34. IET parameters for the reproducibility experiments with the TRIS-ASP-PVA membranes synthesized by Experimenter 2.

Batch	Log (c_{base} / c_{acid})	IET run time / min	Initial IET voltage/ V	Final IET voltage / V	Initial IET current / mA	Final IET current / mA	CE method	IET final compart.
1	0.3	45	350	400	195	89	2	ASC
	0.2	50	400	450	207	103	2	CSC
2	0.3	50	400	450	183	97	2	ASC
	0.2	50	400	450	179	100	2	CSC
3	0.3	55	450	450	211	91	2	ASC
	0.2	55	400	400	229	79	2	CSC

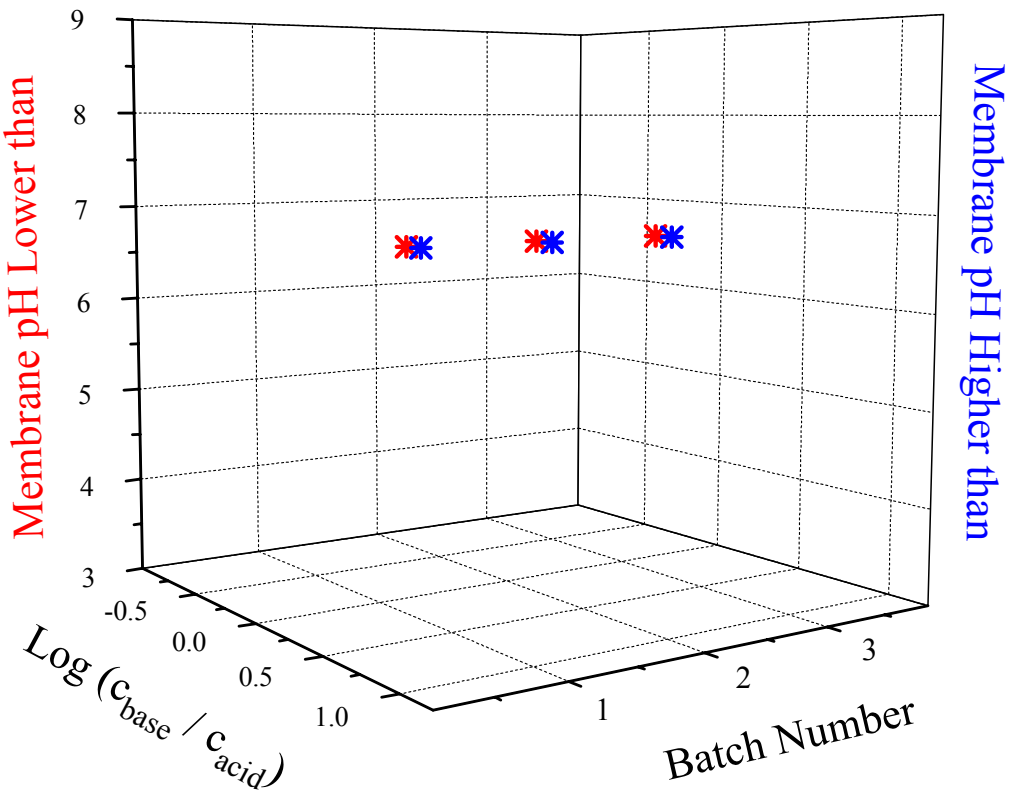


Figure 33. Graph of membrane pH versus composition, expressed as log (c_{base} / c_{acid}) for the three different batches in the reproducibility experiments with the TRIS-ASP-PVA membranes synthesized by Experimenter 1.

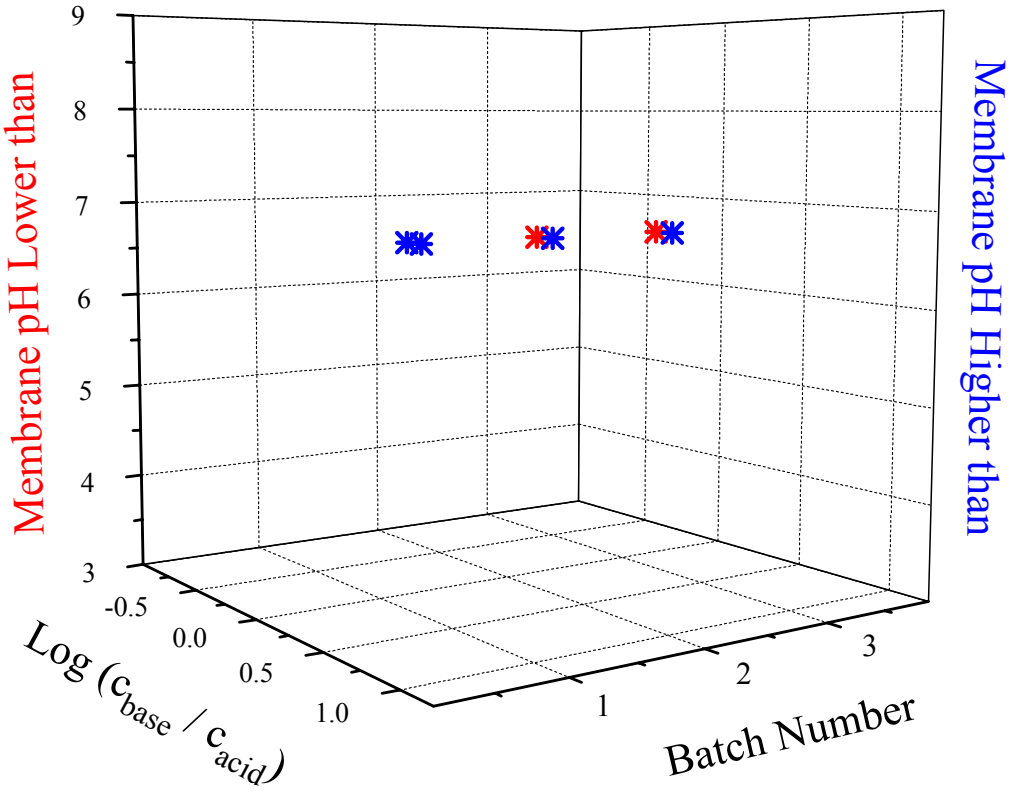


Figure 34. Graph of membrane pH versus composition, expressed as log (c_{base} / c_{acid}) for the three different batches in the reproducibility experiments with the TRIS-ASP-PVA membranes synthesized by Experimenter 2.

4. ISOELECTRIC TRAPPING AND DESALTING EXPERIMENTS WITH TUNABLE PVA-BASED BUFFERING MEMBRANES

4.1 Isoelectric trapping of small ampholytes with tunable PVA-based membranes

4.1.1 IET Set-up

The utility of the new tunable PVA membranes for isoelectric trapping was tested using the BF200IET in recirculating mode, in single compartment configuration [19] as described in Section 2, in three experiments that probed, pairwise, one membrane from each of the six membrane families developed. The first IET experiment had an ASP-PROP-PVA membrane as the anodic membrane, with a composition corresponding to log (c_{base} / c_{acid}) = 0.2 (approximate membrane pH = 4.9), and a TRIS-ASP-PVA membrane as the cathodic membrane with a composition corresponding to log (c_{base} / c_{acid}) = 0.6 (approximate membrane pH = 7.1). The second IET experiment had a MORPH-ASP-PVA membrane as the anodic membrane, with a membrane composition corresponding to log (c_{base} / c_{acid}) = 1.0 (approximate membrane pH = 6.6), and a PROP-ASP-PVA membrane with a composition corresponding to log (c_{base} / c_{acid}) = 1.0 as the cathodic membrane (approximate membrane pH = 8.6). The third experiment was done with a MAL-PROP-PVA membrane as the anodic membrane, with a membrane composition corresponding to log (c_{base} / c_{acid}) = 0.5 (approximate membrane pH = 4.0), and a DEAPA-ASP-PVA membrane with a composition corresponding to log (c_{base} / c_{acid}) = 1.0 as the cathodic membrane (approximate membrane pH = 10). For all experiments, the anolyte contained 30 mM methanesulfonic acid and 10 mM para-

toluenesulfonic acid, and the catholyte contained 60 mM NaOH (30 mM NaOH in the first experiment) and 10 mM benzyltrimethylammonium hydroxide. The UV-absorbing acids and bases were added to determine if there was any acid or base invasion into the sample compartment during IET. Initially, the sample compartment contained a 2 mM solution of a pI marker. Isoelectric trapping was carried out for 3 hours. The pI marker used, the initial IET potential, the final IET potential, the initial IET current, and the final IET current for all three experiments are listed in Table 35. Aliquots were taken from the sample compartment stream every 1 hour and analyzed by CE using method 3 from Table 12.

Table 35. The pI marker used, the initial and final IET potential, and the initial and final IET current for the three IET experiments.

Experiment	pI marker	Initial IET Potential / V	Final IET Potential / V	Initial IET Current / mA	Final IET Current / mA
1	MK-6.7	400	400	87	61
2	MK-7.6	400	400	92	49
3	MK-6.7	400	400	100	57

4.1.2 Results

The CE traces for the aliquots taken from the sample compartment of the BF200IET at 1 hour, 2 hours, and 3 hours for the three experiments are shown in Figures 35, 36, and 37. In all three cases, using representative membrane pairs from all of the six new membrane families, the pI marker has remained in the sample compartment, and none of the UV-absorbing acid or base invaded the sample compartment.

4.2 Desalting experiments with tunable PVA-based membranes

4.2.1 Background and objectives

Most protein samples contain a large amount of salts, originating either from their sources or added to aid protein solubility and/or stability. The presence of a high salt concentration in a protein sample can be undesirable for certain downstream analyses; therefore desalting of protein samples is a major concern. The current desalting techniques include dialysis methods, gel filtration methods, and protein precipitation. These techniques typically take a long time, and can often lead to protein loss, especially for low molecular weight and/or low concentration proteins. This can be detrimental in proteomics analyses since analysis of the entire proteome is desired. IET has been used as an alternative desalting technique because it provides fast and efficient removal of strong electrolytes without sample loss [19, 30, 31, 46].

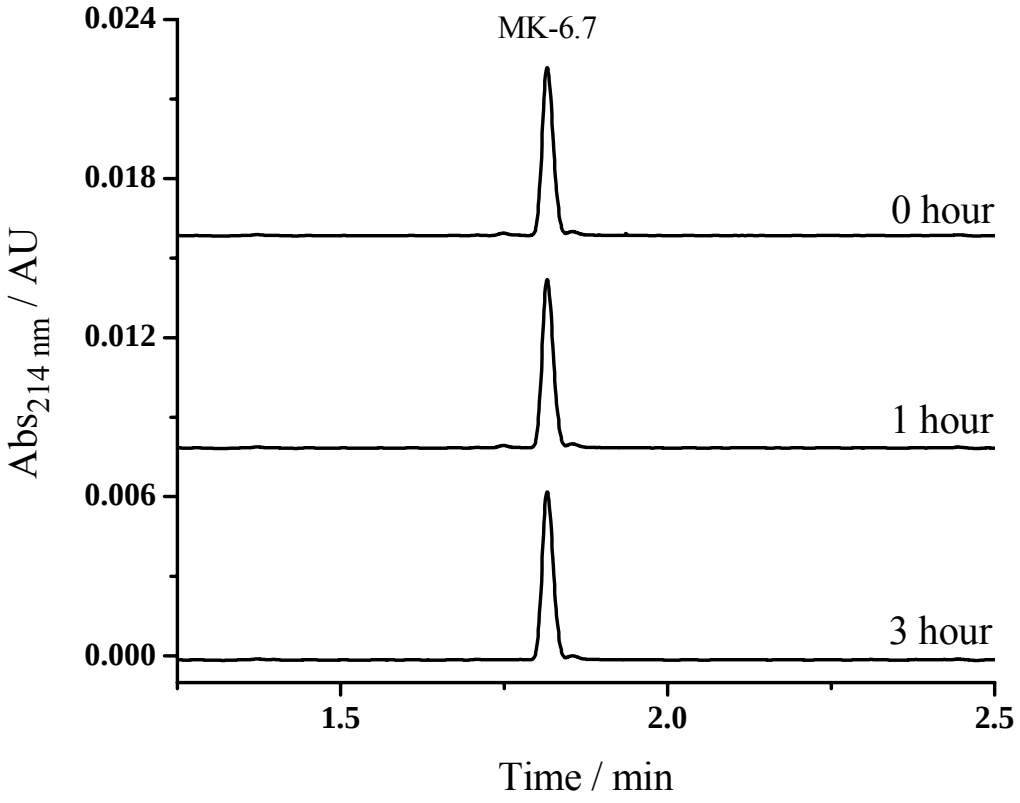


Figure 35. Electropherograms of the samples taken from the first IET experiment with ASP-PROP-PVA as the anodic membrane and TRIS-ASP-PVA as the cathodic membrane.

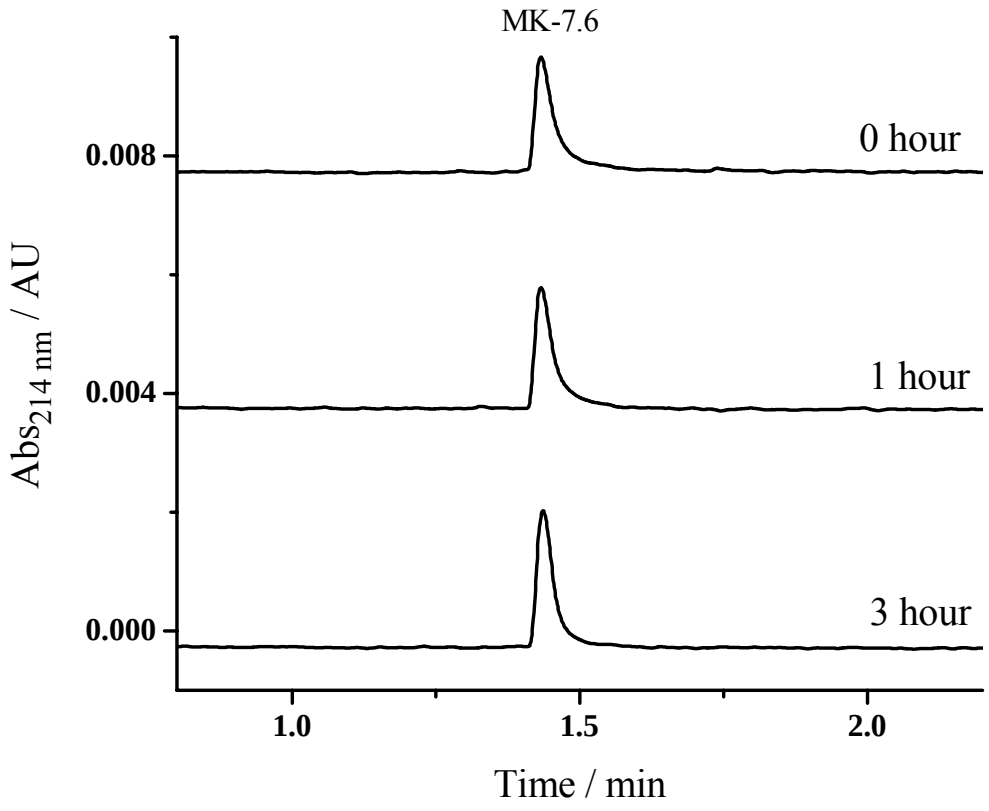


Figure 36. Electropherograms of the samples taken from the second IET experiment with MORPH-ASP-PVA as the anodic membrane and PROP-ASP-PVA as the cathodic membrane.

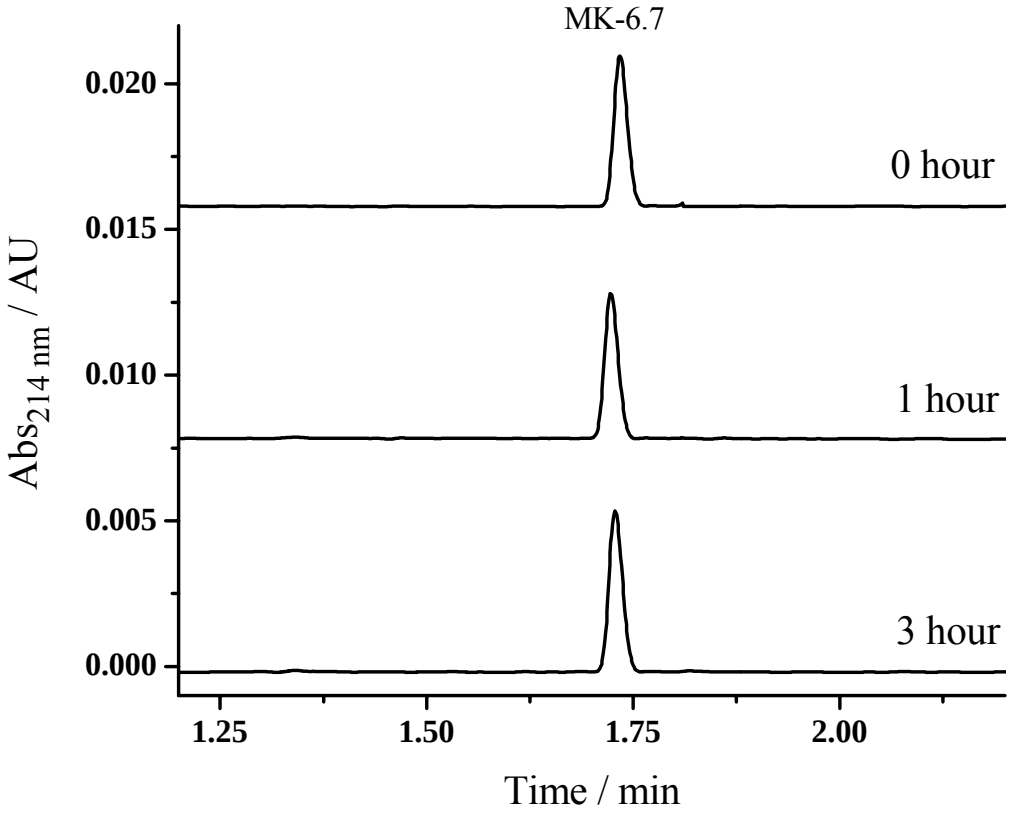


Figure 37. Electropherograms of the samples taken from the third IET experiment with MAL-PROP-PVA as the anodic membrane and DEAPA-ASP-PVA as the cathodic membrane.

4.2.2 pH transients in IET desalting experiments

In IET desalting experiments, pH transients can result because of the unequal removal rates of strong electrolytes. The resulting acidic or basic conditions can have a detrimental effect on proteins. A recent paper discusses how pH transients in IET depend on the mobilities of strong electrolytes (anions and cations) and the pH values of the buffering membranes [46]. It was found that when the pH values of the anodic and cathodic membranes in a single compartment IET set-up are equidistant from 7, the direction of the pH transient (acidic or basic) is determined by the mobilities of the anion and cation (μ_{anion} and μ_{cation}). When $\mu_{anion} / \mu_{cation} > 1$, an acidic transient occurs, and when $\mu_{anion} / \mu_{cation} < 1$, a basic transient occurs. In addition, the direction of the pH transient also depends on the pH of the membranes. When $\mu_{anion} / \mu_{cation} = 1$ and $(\left| pH_{anodic\ membrane} - 7 \right| / \left| 7 - pH_{cathodic\ membrane} \right|) > 1$, there will be an acidic transient. When $\mu_{anion} / \mu_{cation} = 1$ and $(\left| pH_{anodic\ membrane} - 7 \right| / \left| 7 - pH_{cathodic\ membrane} \right|) < 1$, there will be a basic transient. When $\mu_{anion} / \mu_{cation} = 1$ and $\left| pH_{anodic\ membrane} - 7 \right| = \left| 7 - pH_{cathodic\ membrane} \right|$, there will be no pH transient. With careful selection of the buffering membranes, pH transients can be avoided when $\mu_{anion} / \mu_{cation} \neq 1$ by finding the appropriate $(\left| pH_{anodic\ membrane} - 7 \right| / \left| 7 - pH_{cathodic\ membrane} \right|) \neq 1$ value.

4.2.3 IET Set-up

Desalting experiments were performed using a modified BF200IET described in Section 4.1, in the single compartment configuration, operated in recirculating mode. Fractions were collected from all streams at pre-set times and analyzed by CE. The pH values of

the samples were measured with a glass microelectrode MI-414 (Microelectrodes, Inc., Bedford, NH, USA) and a Corning Model 150 pH meter (Corning, Inc., Corning, NY, USA). The specific conductivities of the samples were measured with a MI-905 conductivity microprobe (Microelectrodes, Bedford, NH, USA) and a model 145+ conductivity meter (Thermo-Orion, Beverly, MA, USA).

Three IET desalting experiments were run, with the same anodic and cathodic membranes as used in the IET experiments described in Section 4.1. In all three desalting experiments, the anolyte contained 30 mM methanesulfonic acid, and the catholyte contained 60 mM NaOH (30 mM NaOH for the first experiment). The solution in the sample compartment contained a pI marker at a concentration of 2 mM, and a UV-absorbing salt, benzyltrimethylammonium para-toluenesulfonate ($BzTMA^+$ PTS^-), at a concentration of 10 mM. Isoelectric trapping was carried out for 60 minutes, and aliquots were taken from the sample compartment stream every 2 minutes and analyzed by CE using method 3 from Table 12. The pI marker used, the initial IET potential, the final IET potential, the initial IET current, and the final IET current for all three desalting experiments are listed in Table 36.

4.2.4 Results

The CE traces for the four aliquots taken from the sample stream in the three desalting experiments are shown in Figures 38, 39, and 40. By 50-60 minutes, $BzTMA^+$ and PTS^- have been completely removed from the sample compartment, but the pI marker

Table 36. The pI marker used, the initial and final IET potential, and the initial and final IET current for the three IET desalting experiments.

Experiment	pI marker	Initial IET Potential / V	Final IET Potential / V	Initial IET Current / mA	Final IET Current / mA
1	MK-6.7	11	400	250	56
2	MK-7.6	12	400	250	45
3	MK-6.7	12	400	250	63

remained trapped. The pH and specific conductivity versus IET time curves for the three experiments are plotted in Figures 41, 42 and 43. As the salt leaves the sample compartment, the conductivity decreases to a final, stable value. In the first experiment, there is an acidic pH transient due to $\left| pH_{\text{anodic membrane}} - 7 \right| / \left| 7 - pH_{\text{cathodic membrane}} \right| > 1$. In the second experiment, there is a basic pH transient because $\left| pH_{\text{anodic membrane}} - 7 \right| / \left| 7 - pH_{\text{cathodic membrane}} \right| < 1$. In the third experiment, $\left| pH_{\text{anodic membrane}} - 7 \right| = \left| 7 - pH_{\text{cathodic membrane}} \right| = 3$, therefore there is no pH transient.

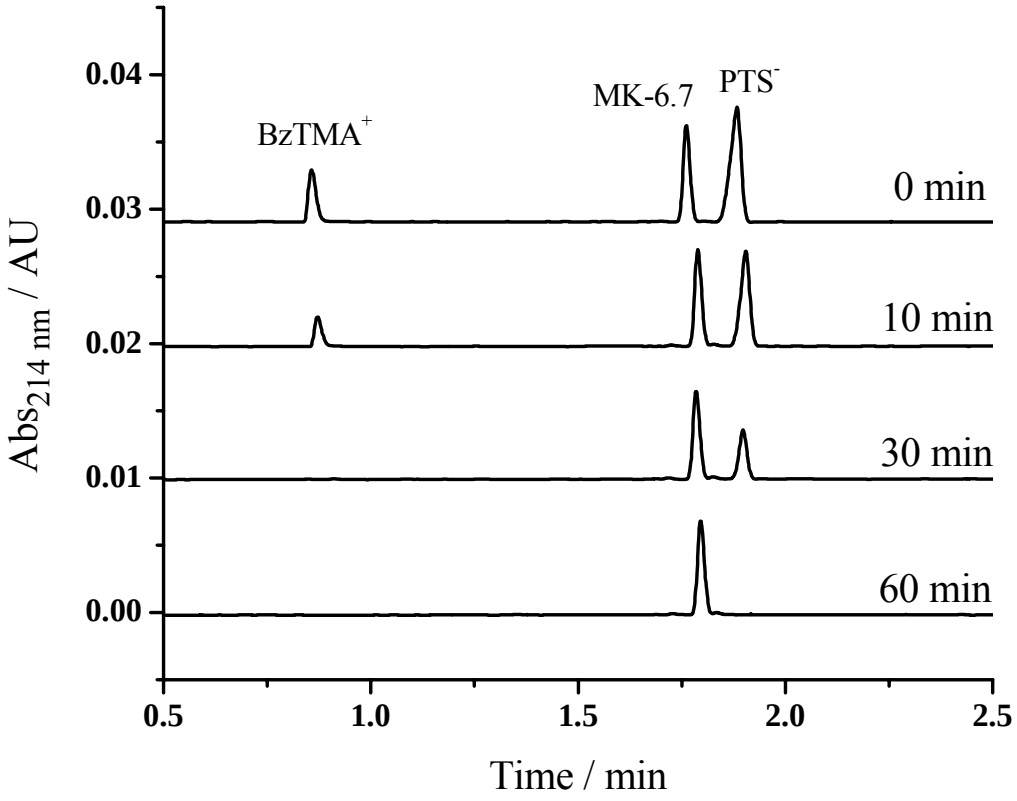


Figure 38. Electropherograms of the samples taken from the first IET desalting experiment with ASP-PROP-PVA as the anodic membrane and TRIS-ASP-PVA as the cathodic membrane.

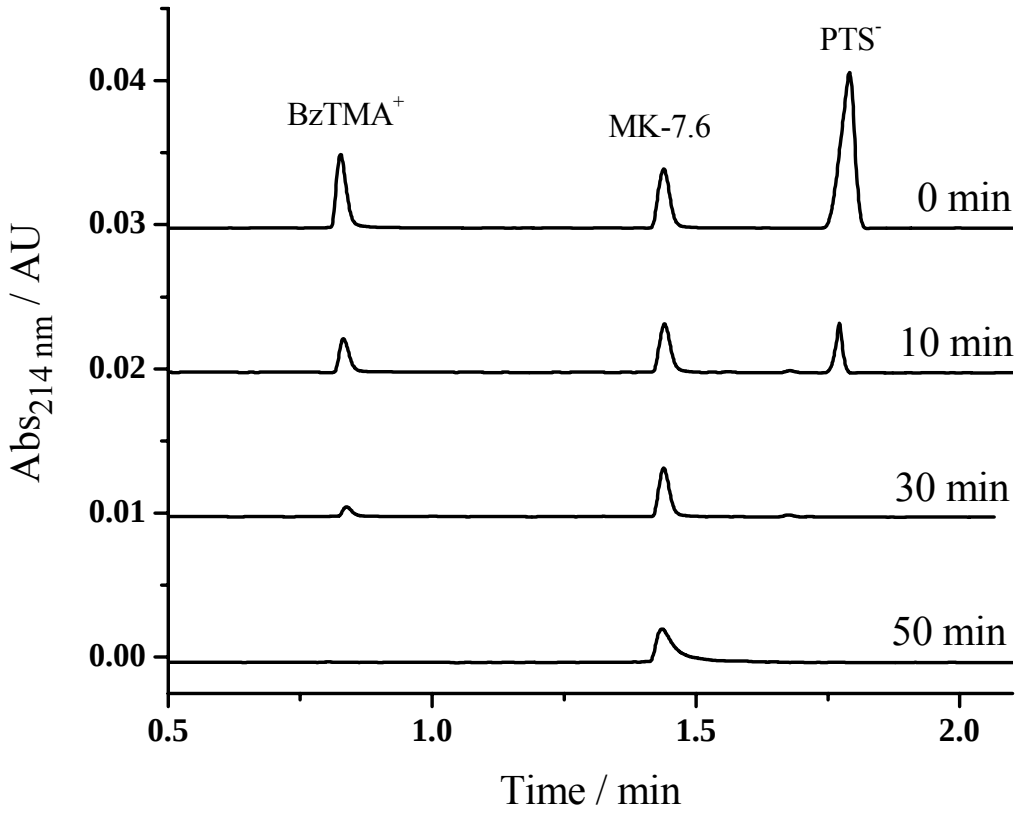


Figure 39. Electropherograms of the samples taken from the second IET desalting experiment with MORPH-ASP-PVA as the anodic membrane and PROP-ASP-PVA as the cathodic membrane.

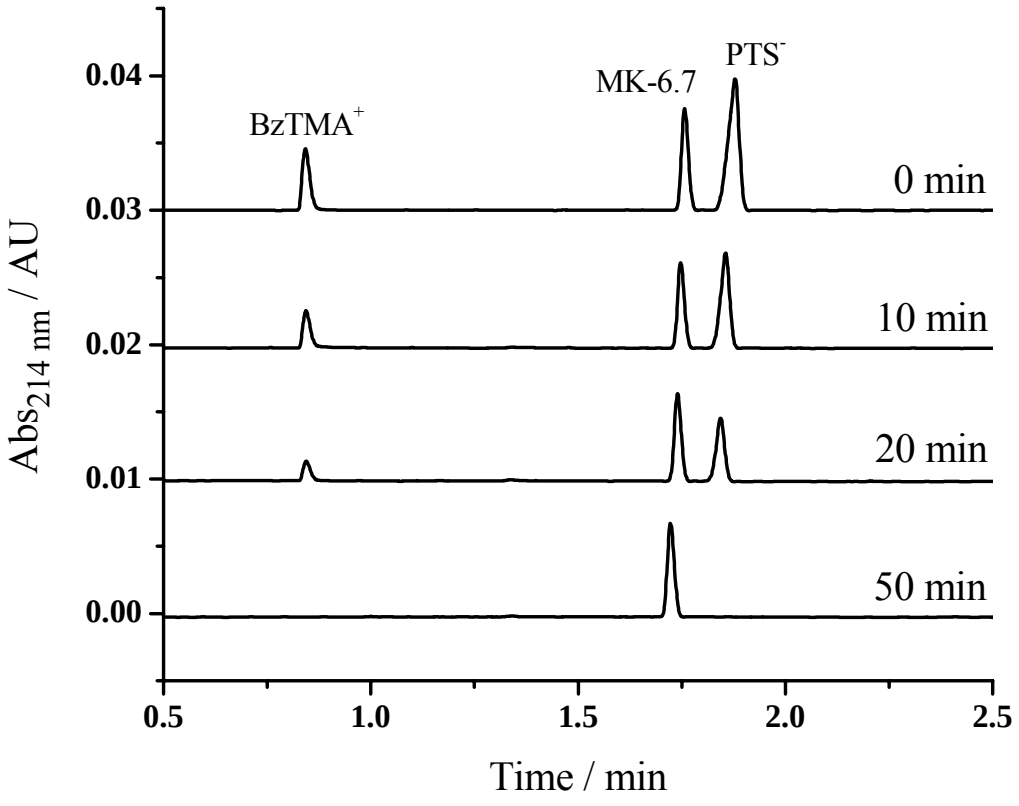


Figure 40. Electropherograms of the samples taken from the third IET desalting experiment with MAL-PROP-PVA as the anodic membrane and DEAPA-ASP-PVA as the cathodic membrane.

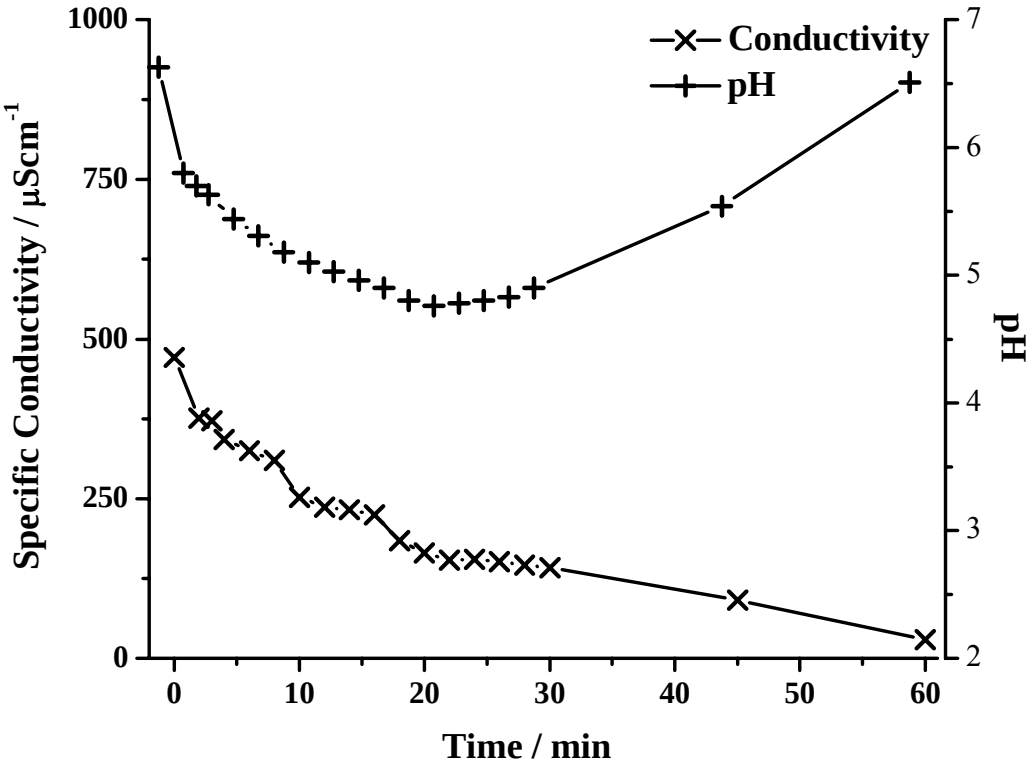


Figure 41. Specific conductivity and pH (versus IET time) of the recirculated solution during the first IET desalting experiment with ASP-PROP-PVA as the anodic membrane and TRIS-ASP-PVA as the cathodic membrane.

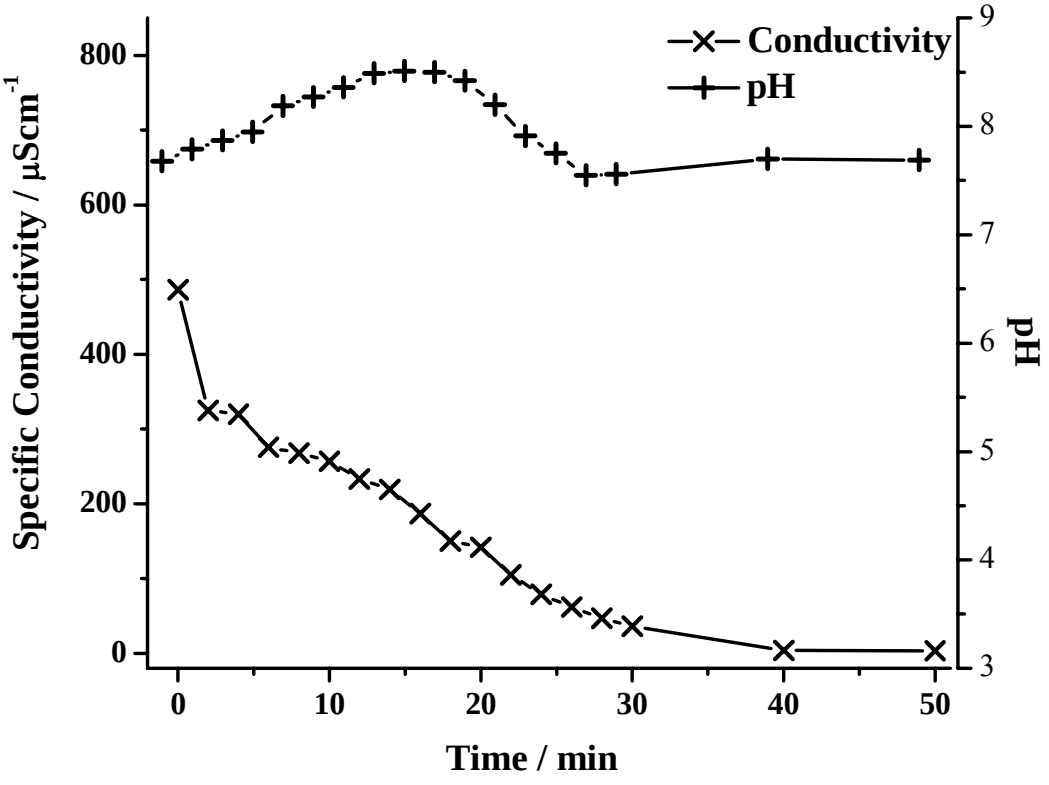


Figure 42. Specific conductivity and pH (versus IET time) of the recirculated solution during the second IET desalting experiment with MORPH-ASP-PVA as the anodic membrane and PROP-ASP-PVA as the cathodic membrane.

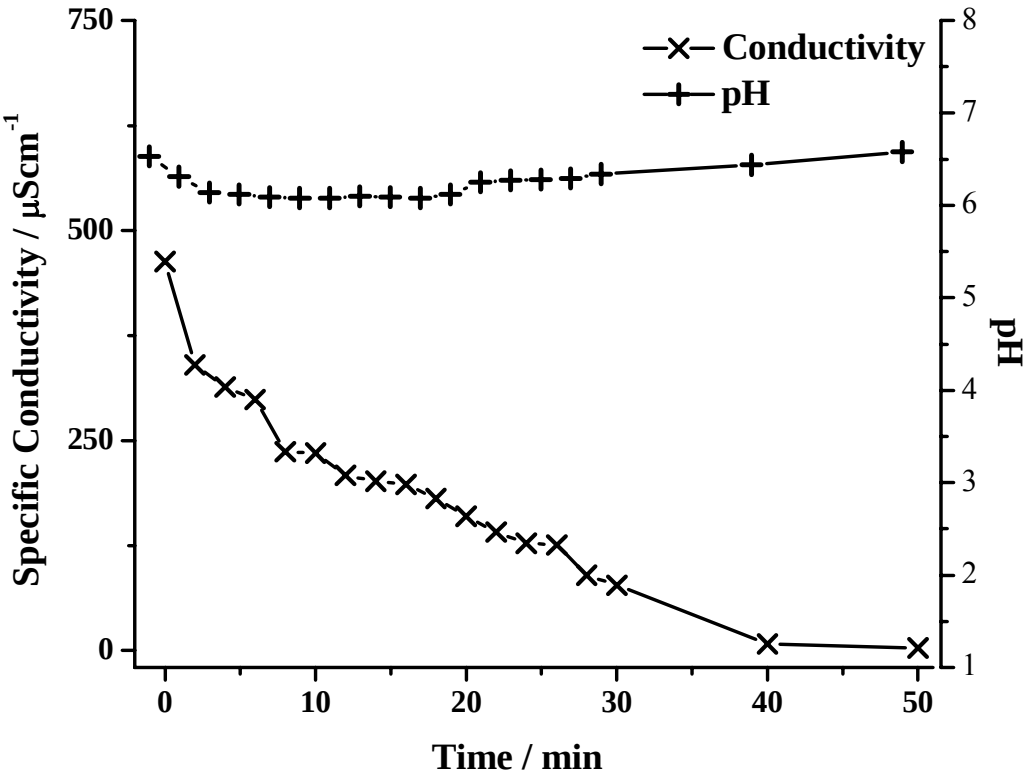


Figure 43. Specific conductivity and pH (versus IET time) of the recirculated solution during the third IET desalting experiment with MAL-PROP-PVA as the anodic membrane and DEAPA-ASP-PVA as the cathodic membrane.

5. PROTEIN SEPARATIONS WITH TUNABLE PVA-BASED BUFFERING MEMBRANES

5.1 Objectives and rationale

The tunable PVA-based membranes were successfully used in the IET separations of small molecular weight compounds. Since IET is commonly used as a protein separation and prefractionation technique, the tunable membranes needed to be tested as separation membranes in protein IET experiments.

5.2 Separation of a large molecular weight protein

Bovine IgG (molecular weight ~ 150 kDa) was used to demonstrate that the tunable pH PVA membranes can be used for the IET separations of large proteins. The BF200IET was set up in the two separation compartment mode [19]. The feed solution that was loaded in the anodic separation compartment contained 1 mg / mL bovine IgG (Equitech-Bio, Kerrville, TX) and 10mM IDA as a pH biaser [20] in 30 mL deionized water. The cathodic separation compartment contained 10 mM of arginine as a biaser in 30 mL deionized water. The anodic membrane was IDA-PVA, the separation membrane was an ASP-PROP-PVA membrane with an approximate pH of 4.0, and the cathodic membrane was Q-PVA. The anolyte was 30 mM methanesulfonic acid, and the catholyte was 180 mM sodium hydroxide. The IET separation was carried out at a constant current of 250 mA, and the potential started at 180 V and finished at 575 V. Samples were taken from both streams every 30 min. The IDA and arginine pH biasers

were removed from the collected samples using an Ultrafree®-MC Centrifugal Filter Unit (Millipore, Billerica, MA, USA) with a molecular weight cut-off of 10 kDa.

The biaser-free solutions were then analyzed by capillary SDS capillary gel electrophoresis using the PA 800 System (Beckman Coulter, Inc., Fullerton, CA, USA) and the ProteomeLab^{TM} SDS-MW analysis kit, Part Number 390953 (Beckman Coulter, Inc.), under non-reducing conditions using the IgG Purity/Heterogeneity Standard Operating Protocol [62]. The 50 μm I.D. bare-fused silica capillary had an injector-to-detector length of 30 cm and total length of 40 cm. The capillary was preconditioned by rinsing at 50 psi with 0.1 N NaOH for 5 min, and 0.1 N HCl for 2 min. The capillary was then filled with the ProteomeLab^{TM} SDS-MW gel (Beckman Coulter, Inc.) for 10 min at 40 psi, and electrophoresed for 10 min at 15 kV. The samples were prepared by mixing 150 μL of the fractions collected during the IET separation with 50 μL of the ProteomeLab^{TM} SDS-MW Sample Buffer (Beckman Coulter, Inc.), 4 μL of the ProteomeLab^{TM} Internal Standard (approximate relative molecular mass 10 kDa), and 10 μL of a 250 mM iodoacetamide solution (to alkylate the protein [59]). The solution was centrifuged at 300 g for 1 min, and then heated at 70º C in a water bath for 10 min and allowed to cool to room temperature for 3 min. A 100 μL portion of the solution was transferred into a microvial for PA 800 analysis, and the samples were injected for 20 s by 5kV. The separations were carried out for 40 min at 15 kV, at 25^{o} C, and the proteins were detected at 220 nm.

The electropherograms of the samples taken from the ASC (feed stream) and CSC (collection stream) at 0 min and 120 min are shown in Figure 44. The top panel shows the initial contents of the anodic separation compartment at 0 min with the bovine IgG present. The middle panel shows the contents of the cathodic separation compartment at the end of the IET separation indicating that IgG has migrated into the CSC. The bottom panel shows the contents of the anodic separation compartment at the end of the IET run. Clearly, all of the bovine IgG has migrated through the separation membrane and was collected in the cathodic separation compartment. The peak areas for bovine IgG in the initial sample and final sample are equal, indicating that the protein did not precipitate on the membrane, and that the pores in the membrane were large enough to allow the passage of the 150 kDa protein.

5.3 Protein separations using the tunable PVA-based buffering membranes

To demonstrate that each series of tunable PVA-based buffering membranes can be used in IET separations of protein mixtures, a membrane from each series was used in an IET experiment. The protein samples were chosen based on the operational membrane pH range for that series.

5.3.1 Separation of ovalbumin isoforms using a MAL-PROP-PVA membrane

Chicken egg white is a complex mixture of proteins with various molecular weights and pI values. The two most abundant proteins in chicken egg white are ovalbumin and ovotransferrin, comprising of 54%, and 12-13% of the total protein content,

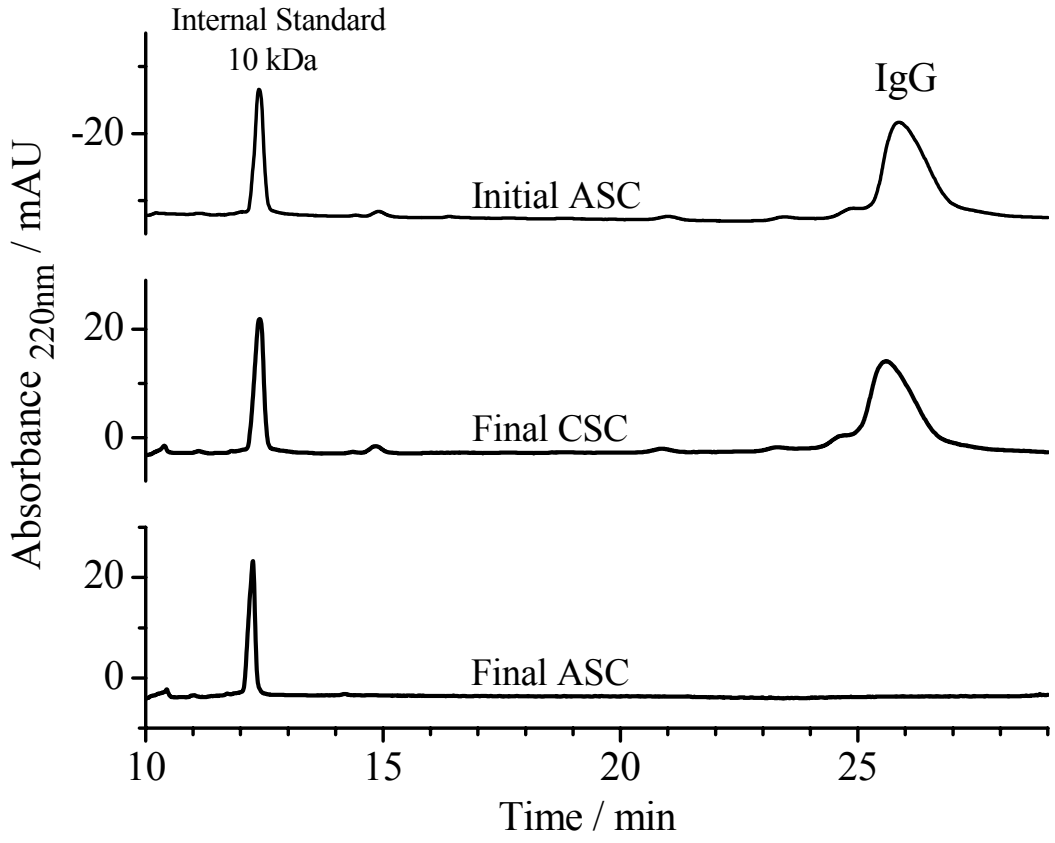


Figure 44. Electropherograms of the samples taken during the bovine IgG separation experiment. Top panel: Sample from the ASC at the start of the IET separation (feed); middle panel: sample from the CSC at the end of the IET separation; bottom panel: sample from the ASC at the end of the IET separation.

respectively[60, 63, 64]. Each of these major proteins has several isoforms that vary slightly from one another in pI value. Table 37 lists some of the major and minor proteins in chicken egg white, their percent content (w/w) of the total proteins in egg white, their molecular weight, and the pI value range of the isoforms.

Table 37. Some major and minor proteins in chicken egg white, their percent content, molecular weight, and pI value.

Protein	% of Total Proteins	MW (kDa)	pI
Ovomucoid	11	28	3.83-4.41
Ovalbumin	54	45	4.75-4.94
Ovoglobulin	8	49	5.5-5.8
Ovotransferrin	12-13	77.7	6.2-7.2
Lysozyme	3.5	14.3	10.5

The BF200IET was used to demonstrate the separation of ovalbumin isoforms using a MAL-PROP-PVA membrane (calculated pH = 4.7) as the separation membrane. In this experiment, IDA-PVA was used as the anodic membrane, and LYS-PVA was used as the cathodic membrane. The anolyte was 30 mM methanesulfonic acid and the catholyte was 60 mM NaOH. Twenty-five mL of 1 mg / mL sample of ovalbumin from chicken

egg white (Sigma, St. Louis, MO) was added to the cathodic separation compartment. The anodic separation compartment originally contained 25 mL of a 10 mM solution of IDA used as a pH biaser [20]. The IET separation was carried out for 60 minutes with an initial potential of 76 V, and an initial current of 250 mA. The potential increased to 400 V, and the current decreased to 81 mA by the end of the run.

Fractions were collected from both sample streams and analyzed using full-column imaging IEF with an iCE280 unit (Convergent Biosciences, Toronto, Canada) connected to an HPLC autosampler (Alcott Chromatography, Inc., Norcross, GA, USA). The separation cartridge in the iCE280 unit contained a fluorocarbon-coated fused-silica capillary with an internal diameter of 100 μm and a separation length of 5 cm. Eighty mM phosphoric acid containing 0.1 % w/w methyl cellulose was used as the anolyte, and 100 mM NaOH containing 0.1 %w/w methyl cellulose was used as the catholyte. Methyl cellulose was used to suppress electroosmotic flow in the capillary during the IEF separation. A 20 μL aliquot of the samples taken from the anodic and cathodic separation compartments of the BF200IET was added to 80 μL of a mixture containing 4 %v/v carrier ampholyte 3-10 (Pharmacia), arginine and IDA as pH blockers, pI markers MK-3.2, and MK-8.3, and 0.35 %w/w methyl cellulose. The pI markers were used to calibrate the pH gradient formed by the carrier ampholytes in the capillary. IEF was carried out for 30 seconds at 500 V and then for 6 minutes at 3000 V.

Figure 45 shows the IEF traces of the initial sample from the CSC (feed), and the final CSC and ASC samples. Clearly, the ovalbumin isoforms have been separated. The more acidic isoforms migrated into the ASC, while the more basic isoforms remained in the CSC.

5.3.2 Binary separation of proteins in chicken egg white using an ASP-PROP-PVA membrane

The BF200IET unit was used to obtain a binary separation of the major proteins in chicken egg white with an ASP-PROP-PVA membrane as the separation membrane. The two major proteins – ovalbumin and ovotransferrin – were used to follow the course of the separation. An IDA-PVA membrane was used as the anodic membrane, a LYS-PVA as the cathodic membrane, and an ASP-PROP-PVA membrane with a calculated pH of 5.8 as the separation membrane. The anolyte contained 30 mM methanesulfonic acid, and the catholyte contained 60 mM NaOH. Five mL of chicken egg white was diluted into 100 mL of deionized water, and the solution was then filtered through a glass fiber filter membrane. Twenty-five mL of this solution was then placed in the anodic separation compartment of the BF200IET. The cathodic separation compartment originally contained 25 mL of a 10 mM solution of MK-8.1 used as a pH biaser [20]. IET was carried out for 70 minutes. The initial current was 250 mA, and the initial potential was 47 V. By the end of the run the current had decreased to 76 mA and the potential had increased to 400 V.

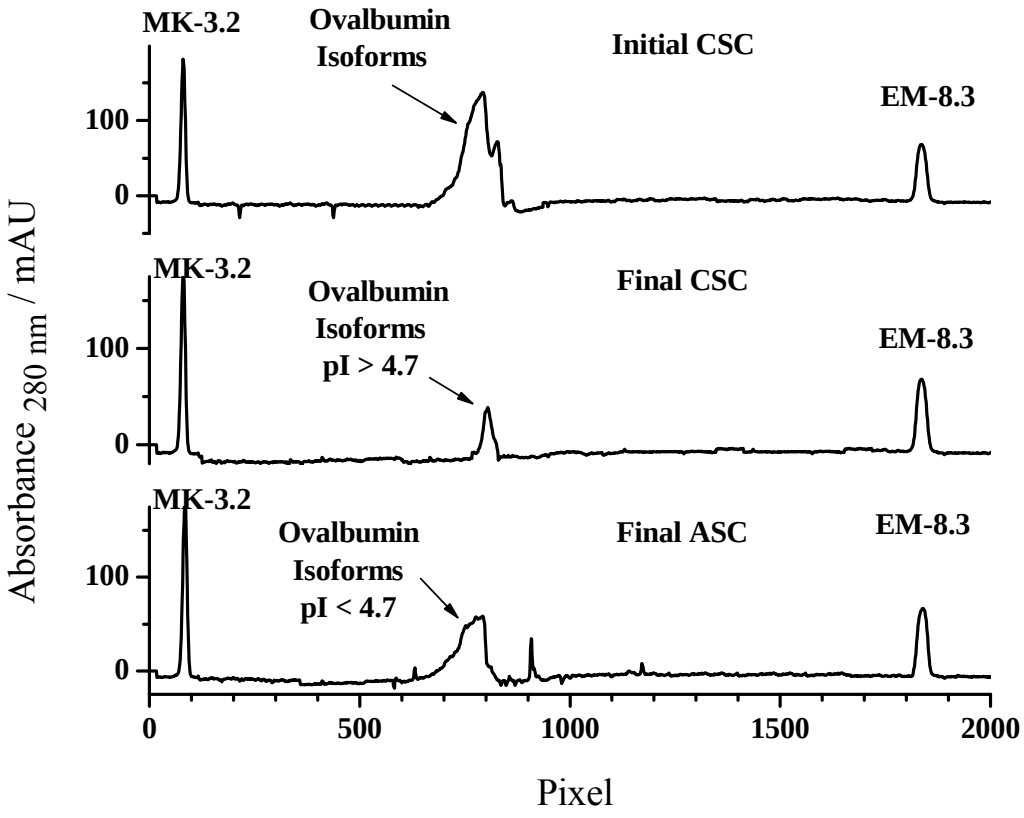


Figure 45. Capillary IEF traces of the samples taken from the IET compartments in the ovalbumin separation. Top trace: initial CSC sample; middle trace: final CSC sample; bottom trace: final ASC sample.

Fractions from both sample compartments were collected every 15 minutes and analyzed by CE. The pH biaser was first removed from the samples taken from the CSC aliquots using an Ultrafree®-MC Centrifugal Filter Unit (Millipore, Billerica, MA, USA) with a molecular weight cut-off of 10 kDa. The CE separations were carried out with a capillary that had a total length of 46.7 cm, an injector-to-detector length of 40 cm, and an internal diameter of 50 µm. The inner surface of the CE capillary used was coated with a crosslinked anionic polymer layer prepared as follows. The inner surface of the bare fused silica capillary was first reacted with 3-(trimethoxysilyl)propyl methacrylate (BindSilane) according to Hjerten's procedure [65]. The immobilized methacrylate groups were then reacted with a mixture of N,N'-methylenebisacrylamide, acrylamide and 80 % 2-acrylamide-2-methylpropanesulfonic acid, in the presence of N,N,N',N'-tetramethylethylenediamine as a catalyst [65]. This produced an anionic coated capillary with an approximate electroosmotic flow of 34×10^{-5} cm^2/Vs [66]. The background electrolyte was a 62 mM ethanolamine solution that was titrated to pH 9.1 with HEPES. The detector wavelength was set to 214 nm, and the applied potential was 10 kV.

The electropherograms for the feed (initial ASC sample), and the final ASC and CSC samples are shown in Figure 46. By the end of the IET separation, all of the ovotransferrin has migrated to the CSC, and the ovalbumin has remained in the ASC, indicating that a binary separation has indeed been achieved with the ASP-PROP-PVA membrane.

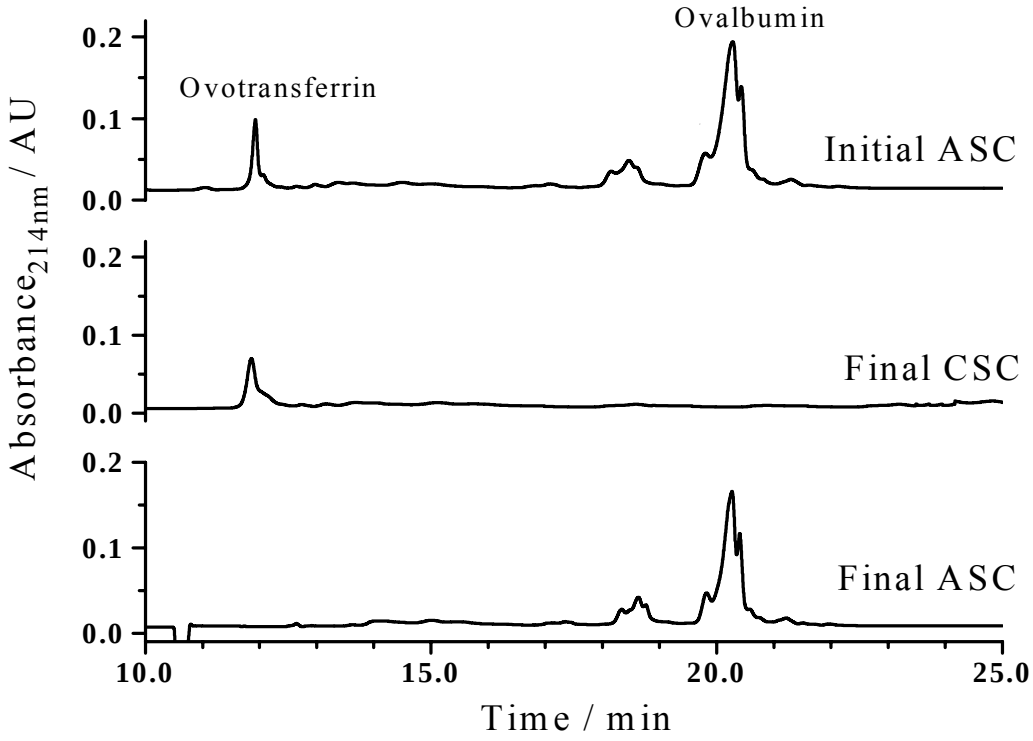


Figure 46. Electropherograms of the samples taken during the binary separation of the proteins in chicken egg white. Top panel: sample taken from the ASC at the start of the IET separation; middle panel: sample taken from the CSC at the end of the IET separation;; bottom panel: sample taken from the ASC at the end of the IET separation.

5.3.3 Separation of the two isoforms of β –lactoglobulin using a MORPH-ASP-PVA membrane

β-lactoglobulin is a major whey protein found in bovine milk. It is a relatively small dimeric protein of 162 residues, with a molecular weight of 18.4 kDa. β -lactoglobulin has two isoforms: A and B. These two isoforms differ by only 2 amino acid residues, and differ in pI value by only 0.1 unit (nominal pI values 5.2 and 5.3) [67]. In order to test the resolving power of the tunable PVA-based buffering membranes, an IET separation was done to separate the two isoforms of bovine β -lactoglobulin.

For the separation the BF200IET unit was used in the two separation compartment mode [19]. IDA-PVA was used as the anodic membrane and LYS-PVA as the cathodic membrane. Three MORPH-ASP-PVA membranes were made, at log (c_{base} / c_{acid}) of -0.6, -0.65, and -0.7, buffering in the vicinity of the nominal pI values of β-lactoglobulin isoforms. Each of the MORPH-ASP-PVA membranes were used as separation membranes in the IET experiment. The anolyte contained 30 mM methanesulfonic acid, and the catholyte contained 50 mM NaOH. The anodic separation compartment initially contained and aqueous solution of 10 mM glutamic acid, and 1 mg / mL β -lactoglobulin from bovine milk (Sigma, St. Louis, MO). The cathodic separation compartments initially contained 10 mM histidine, and 1 mg / mL of the protein. A constant potential of 400 V was applied during IET, and the current went from a typical initial value of 208 mA to a typical final value of 62 mA.

Samples were taken from the anodic and cathodic separation compartments every 15 minutes and analyzed by CE as described in Section 5.3.2. In the IET experiment with MORPH-ASP-PVA, log (c_{base} / c_{acid}) = -0.6 as the separation membrane, both β-lactoglobulin isoforms accumulated in the anodic separation compartment. When the MORPH-ASP-PVA, log (c_{base} / c_{acid}) = -0.7 membrane was used as the separation membrane, both β-lactoglobulin isoforms accumulated in the cathodic separation compartment. When the MORPH-ASP-PVA, log (c_{base} / c_{acid}) = -0.65 membrane was used as the separation membrane, the isoforms were separated into the two sample compartments, as shown in Figure 47. Clearly the latter membrane composition resulted in a membrane pH that was in between the pI values of the two β-lactoglobulin isoforms under the conditions of the IET experiment.

5.3.4 Separation of the two major isoforms of horse heart myoglobin using a TRIS-ASP-PVA membrane

Equine (horse) myoglobin is a globular protein and is 17.5 kDa in size. It has two major isoforms. The more basic isoform (approximate pI = 7.0) is more abundant than the acidic isoform (approximate pI = 6.8) [68]. The tunable PVA-based buffering membranes were used an in IET experiment to separate these two isoforms.

The BF200IET unit was again operated in the two separation compartment mode [19]. The anodic membrane was an IDA-PVA membrane, and the cathodic membrane was a Q-PVA membrane. The separation membrane was a TRIS-ASP-PVA membrane with a

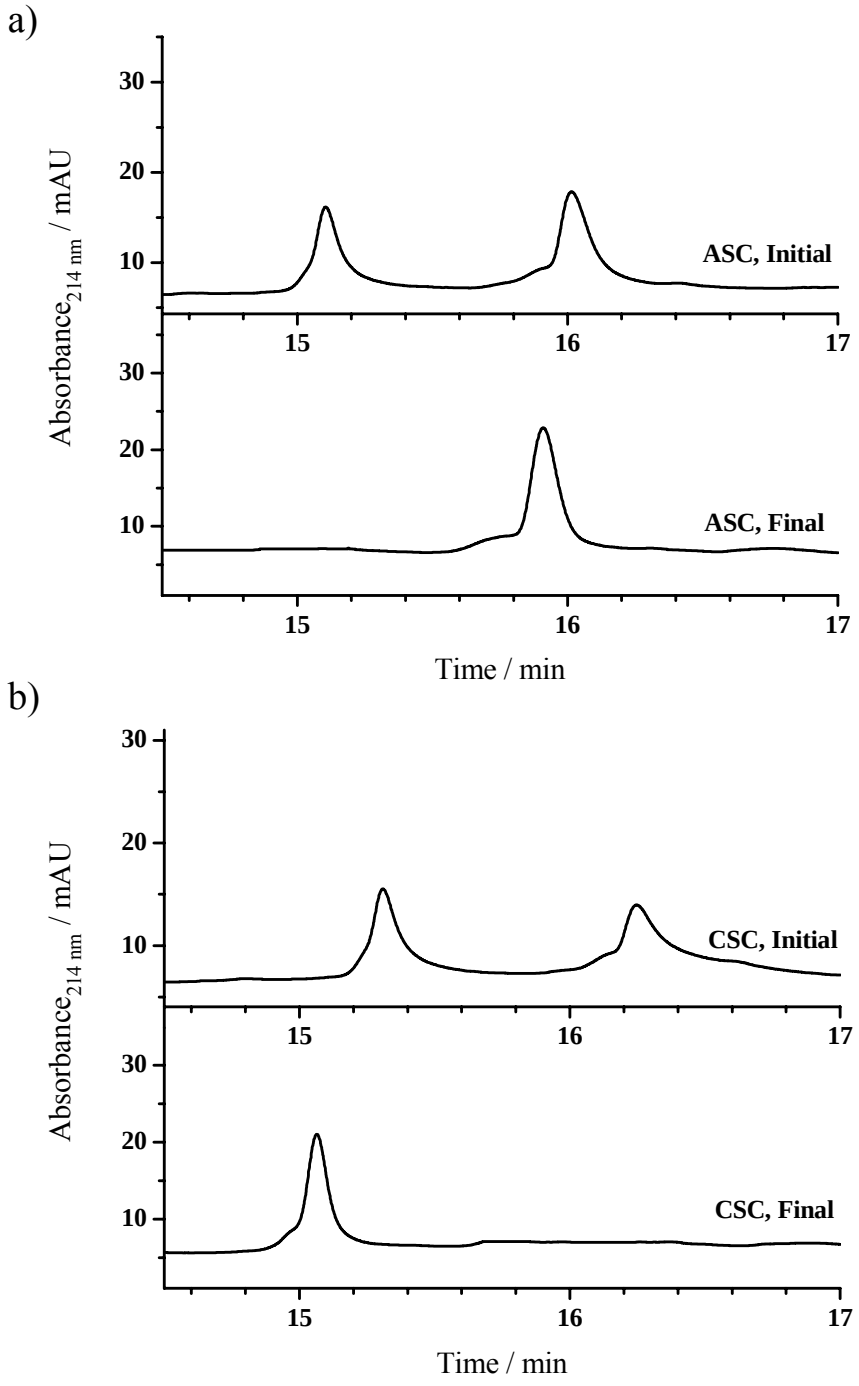


Figure 47. a) Electropherograms of the β-lactoglobulin samples taken from the ASC at the beginning and end of the IET separation. b) Electropherograms of the β-lactoglobulin samples taken from the CSC at the beginning and end of the IET separation.

calculated pH of 6.85. The anolyte was 30 mM methanesulfonic acid, and the catholyte was 180 mM NaOH. The ASC initially contained 30 mL of a solution of 1 mg / mL horse heart myoglobin (Sigma, St. Louis, MO) and 10 mM IDA as a pH biaser [20]. The CSC initially contained 30 mL of a solution of 1 mg / mL horse heart myoglobin and 10 mM arginine as a pH biaser. IET was carried out for 60 minutes, with a constant potential of 375 V. The initial current was 177 mA, the final current 67 mA.

Fractions were collected from both sample streams and analyzed using full-column imaging IEF as described in Section 5.3.1. The capillary IEF traces for the initial and final samples taken from the anodic and cathodic separation compartments of the BF200IET are shown in Figure 48. Initially, both major myoglobin peaks are present. At the end of the IET separation, the minor, acidic isoform is in the anodic separation compartment, and the major, basic isoform is in the cathodic separation compartment.

5.3.5 Separation of isoforms of bovine hemoglobin using a PROP-ASP-PVA membrane

Hemoglobin is an oxygen-carrying metalloprotein found in the red blood cells of most vertebrates. Bovine hemoglobin has a molecular weight of about 64.5 kDa, and has several isoforms with pI values in the 7.0-7.5 range. A PROP-ASP-PVA membrane was used as a separation membrane in IET to separate these isoforms.

The BF200IET unit was again operated in the two separation compartment mode [19]. The anodic membrane was an IDA-PVA membrane, and the cathodic membrane was a

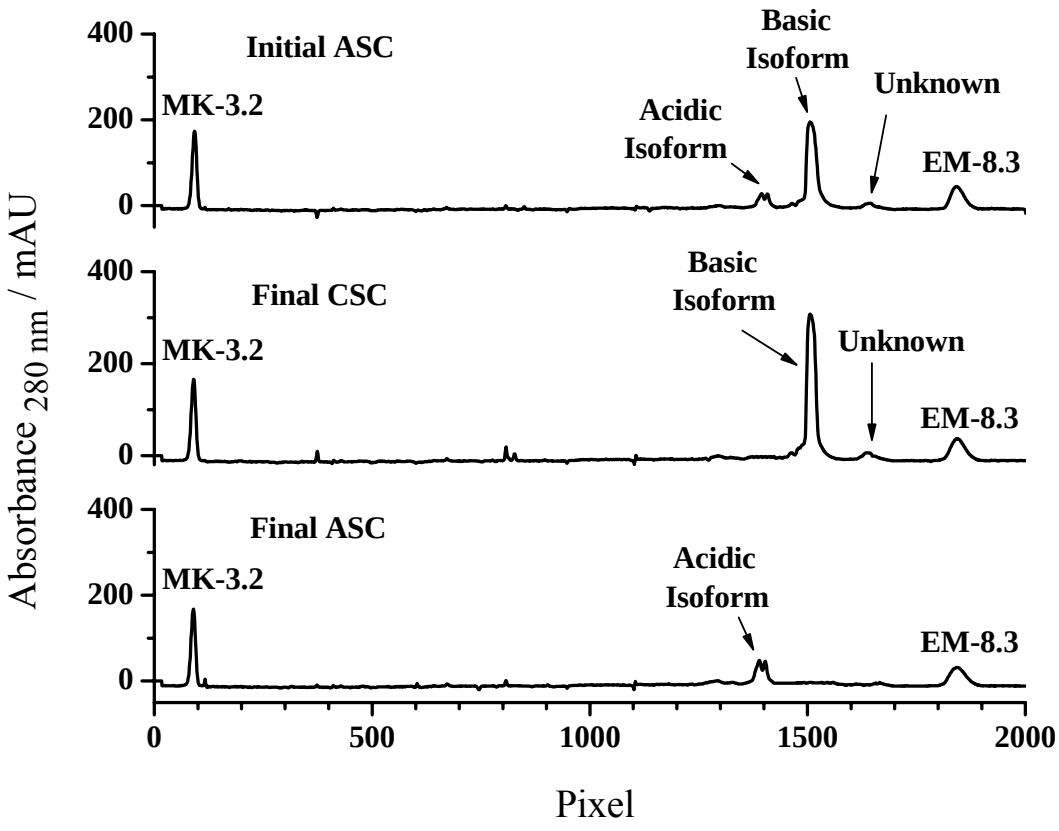


Figure 48. Capillary IEF traces for the samples taken from the anodic and cathodic separation compartments in the IET separation of myoglobin. Top panel: sample from the ASC at the start of the IET separation; middle panel: sample from the CSC at the end of the IET separation; bottom panel: sample from the ASC at the end of the IET separation.

Q-PVA membrane. The separation membrane was a PROP-ASP-PVA membrane with a calculated pH of 7.3. The anolyte was 30 mM methanesulfonic acid, and the catholyte was 180 mM NaOH. The ASC initially contained 25 mL of a solution of 1 mg / mL bovine hemoglobin (Sigma, St. Louis, MO) and 10 mM IDA as a pH biaser [20]. The CSC initially contained 30 mL of 10 mM arginine as a pH biaser. IET was carried out for 60 minutes, with a constant potential of 400 V. The initial current was 152 mA, the final current 73 mA.

Fractions were collected from both sample streams and analyzed using full-column imaging IEF with an iCE280 unit as described in Section 5.3.4. Figure 49 shows the capillary IEF traces of the initial ASC (feed) sample, and the final samples taken from the ASC and CSC. In the initial sample, all of the hemoglobin isoforms are present. At the end of the IET run, it is clear that the more basic (pI > separation membrane pH) isoforms have migrated to the CSC.

5.3.6 Separation and concentration of a minor protein from chicken egg white using a DEAPA-ASP-PVA membrane

Lysozyme is a minor protein found in chicken egg white, comprising of only 3.5 % (w/w) of the total protein content [60]. An IET experiment was performed to test if the PVA-based, tunable-pH buffering membranes could be used to simultaneously separate and concentrate lysozyme from a chicken egg white solution.

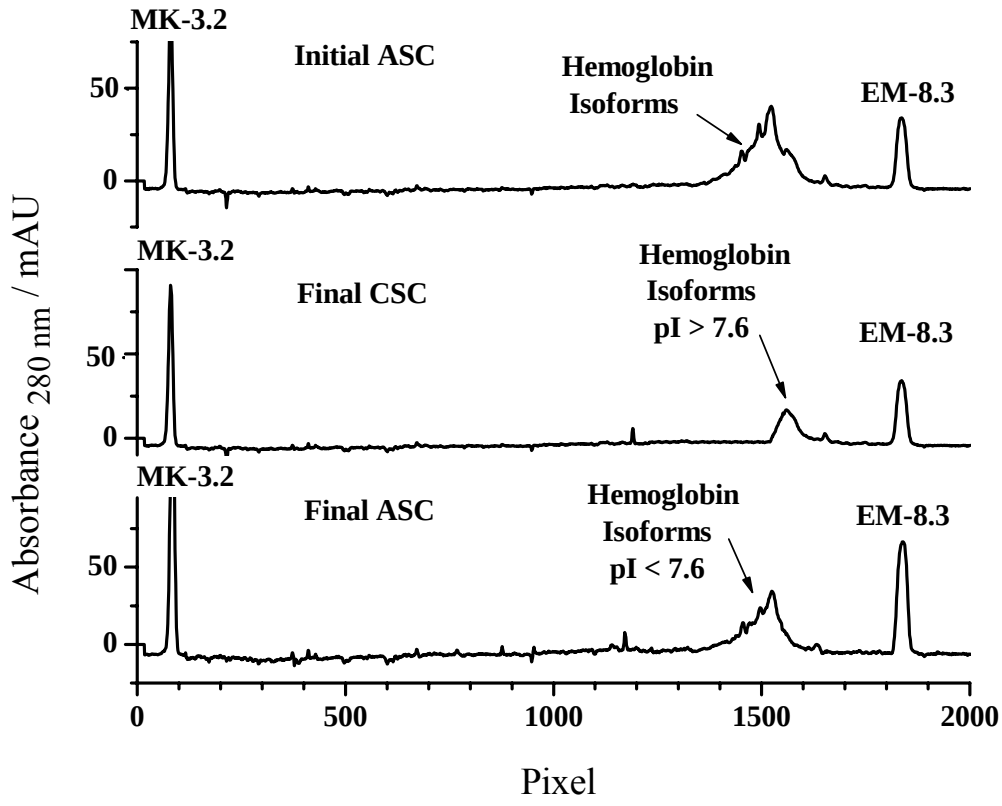


Figure 49. Capillary IEF traces of the samples taken from the samples compartments in the hemoglobin separation. Top panel: initial ASC sample (feed); middle panel: final CSC sample; bottom panel: final ASC sample.

The BF200IET unit was used in the two-compartment operation mode as described in [19]. The anodic membrane was an IDA-PVA membrane, and the cathodic membrane was a Q-PVA membrane. The separation membrane was a DEAPA-ASP-PVA membrane with a calculated pH of 9.0. The anolyte was a 30 mM methanesulfonic acid solution, and the catholyte was a 180 mM NaOH solution. The ASC initially contained 100 mL of a chicken egg white solution diluted 1:20 in deionized water. The CSC initially contained 10 mL of a 10 mM solution of MK-10.0. The proteins that migrated to the CSC would, therefore, be concentrated 10 fold. IET was carried out for 80 minutes. The initial current was set at 300 mA, which resulted in an initial potential of 52 V. At the end of the run, the current decreased to 97 mA, and the potential increased to 400 V.

Samples were collected from the ASC and CSC every 15 minutes and analyzed by SDS capillary gel electrophoresis as described in Section 5.2. Figure 50 shows the electropherograms of the initial ASC sample, and the final ASC and CSC samples. Initially, in the ASC, all of the chicken egg white proteins are present. By the end of the IET separation lysozyme has migrated to the CSC, and has also been concentrated.

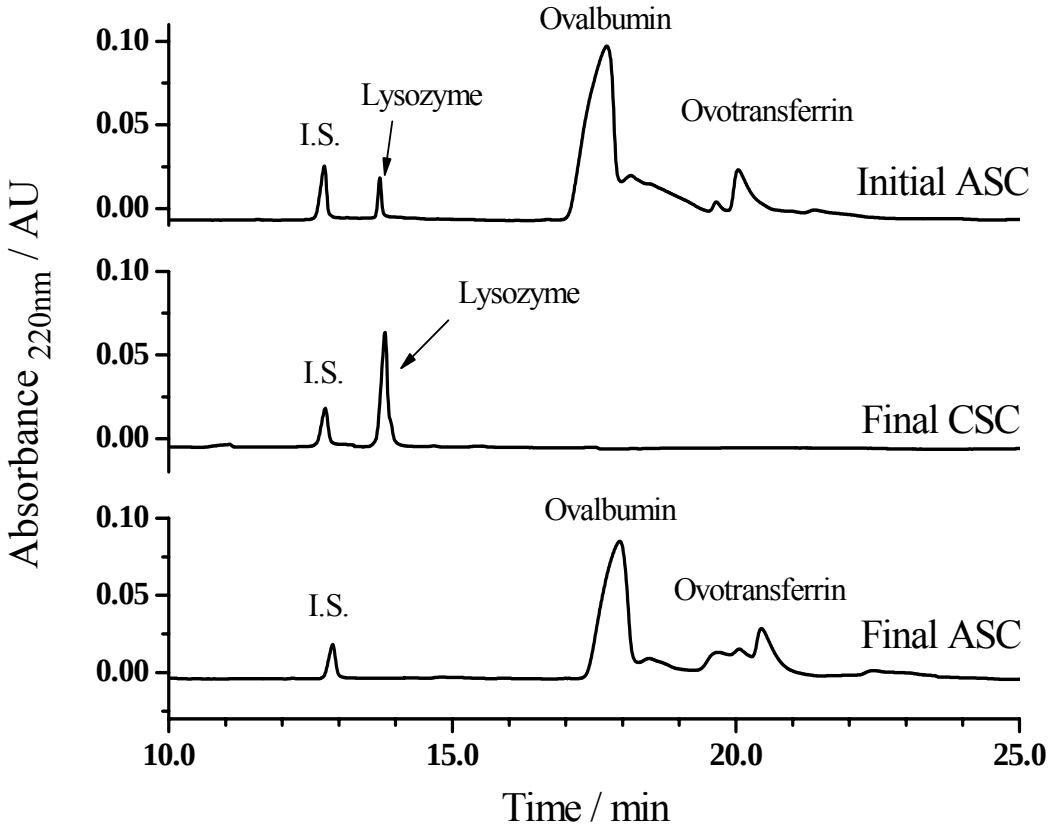


Figure 50. Electropherograms of the samples taken from the IET compartments in the lysozyme separation. Top panel: Sample taken from the ASC at the start of the run; middle panel: sample taken from the CSC at the end of the run; bottom panel: sample taken from the ASC at the end of the run (I.S. = 10 kDa internal standard).

5.4 Concluding Remarks

The PVA-based tunable membranes can be used to separate a wide array of protein samples, including proteins up to 150 kDa. The experiments in this Section showed that these membranes could be used for IET experiments to separate proteins with pI differences as little as 0.1. This is of particular importance in proteomics analyses with complex protein samples that have very closely spaced pI values. In addition, in all of the experiments, there was good protein recovery, indicating that the samples did not precipitate on the membranes, and could easily pass through the separation membrane pores.

6. CONCLUSIONS

6.1 Buffering membranes in IET

IET experiments rely on stable buffering membranes to provide the pH gradient needed for the separations. Due to the hydrolysis of the amide bond, polyacrylamide-based buffering membranes have limited lifetime. Our research group has developed PVA-based low-pH and high-pH buffering membranes for use as anodic and cathodic membranes, respectively, in IET separations. These membranes are hydrolytically and mechanically stable even when exposed to acid and base concentrations as high as 1 M, for extended periods of time.

6.2 Single component PVA-based buffering membranes

Mid-pH PVA-based buffering membranes were made by reacting an ampholytic buffering compound with PVA and GDGE. DAPA-PVA, HIS-PVA, ORN-PVA, and LYS-PVA membranes were made with diaminopropionic acid, histidine, ornithine, and lysine as the ampholyte, respectively. The pH value ranges of these buffering membranes were determined in IET experiments by monitoring the migration direction of pI markers (small compounds with known pI values) through the membranes. The DAPA-PVA and HIS-PVA membranes had an operational buffering pH in the range of 6.3 – 6.6. The ORN-PVA membranes had a pH in the 7.6 – 8.1 range, and the LYS-PVA membranes in the 8.1 – 8.3 range. All four membranes were separately tested as cathodic membranes in trapping and desalting experiments in a modified BF200IET

unit. They were all able to trap pI markers and remove a UV-absorbing salt from a mixture of ampholytes. Additionally, the DAPA-PVA and HIS-PVA membranes were used as separation membranes in a binary separation of major proteins in chicken egg white.

6.3 PVA-based buffering membranes with tunable pH values

The PVA-based membranes described previously can only be tuned stepwise by selecting an ampholytic buffering compound to attach to the PVA matrix. This is not a concern when the membranes are used as anodic and cathodic membranes, however, it presents a problem when they are to be used as separation membranes for experiments that require membranes with finely tuned pH values. Tunable pH PVA-based membranes were therefore synthesized by independently attaching a buffering species and a titrating species to the PVA matrix. Membrane pH was adjusted by varying the concentration ratio of the buffer and titrant. Six series of membranes were synthesized. MAL-PROP-PVA membranes were made using aminomalonic acid as the buffer and 3-amino-1-propanol as the titrant. ASP-PROP-PVA membranes were made using aspartic acid as the buffer and 3-amino-1-propanol as the titrant. MORPH-ASP-PVA membranes were made using 4-(2-aminoethyl)morpholine as the buffer and aspartic acid as the titrant. TRIS-ASP-PVA membranes were made using tris(hydroxymethyl)aminomethane as the buffer and aspartic acid as the titrant. PROP-ASP-PVA membranes were made using 3-amino-1-propanol as the buffer and aspartic

acid as the titrant, and DEAPA-ASP-PVA membranes were made using 3-(diethylamino)propylamine as the buffer and aspartic acid as the titrant.

Several membrane pairs differing by 0.1 in their log (c_{base} / c_{acid}) values were synthesized. The pH values of these membranes were determined by using them as separation membranes in a modified BF200IET unit and monitoring the migration direction, through the membranes, of the pI markers that were placed in the anodic and cathodic separation compartments. The pI value of the marker and its migration direction indicated the highest/lowest pH value a particular membrane could have. The best fit straight line of unit slope drawn through these pH values yielded the operational pK_a value of the buffering group bound to the membrane. The operational pK_a values for the MAL-PROP-PVA, ASP-PROP-PVA, MORPH-ASP-PVA, TRIS-ASP-PVA, PROP-ASP-PVA, and DEAPA-ASP-PVA membranes were 3.82, 4.65, 5.59, 6.48, 7.54, and 8.93, respectively. Using membranes with compositions that yielded $-1 < \log (c_{base} / c_{acid}) < 1$ values, these six membrane series cover the $3 < pH < 10$ range.

When diamines or dicarboxylic acids containing an amino group are used as buffers or titrants in the membranes, additional functional groups are also incorporated that could act as additional buffering components. The amino group in ASP was titrated with 1,3,4,6,7,8-hexahydro-2*H*-pyrimido[1,2-*a*]pyrimidine, or PYR, a stronger base to produce an additional series of membranes. The apparent pK_a value for these membranes is around 9.7, extending the range of the tunable pH membranes even

further. The other amino groups in the MORPH-ASP and DEAPA-ASP mixtures were probed and the apparent pK_a values for both series were determined to be around 7.6. Though these membranes can act as tunable buffering membranes, they do not add to the accessible pH range.

The reproducibility of the membrane synthesis procedure was tested by synthesizing three batches of a pair of membranes (differing by 0.1 in their log (c_{base} / c_{acid}) values) for all six membrane series and repeating the IET pI marker migration experiments. For the 24 membrane pairs tested, 21 pairs directed the pI markers into the expected separation compartments, indicating that the tunable PVA-based membranes can be produced reproducibly. The person-to-person reproducibility of the synthesis procedure was also tested by having two other researchers each synthesize three batches of one pair of membranes. These membranes were then tested in IET using the pI markers. Five out of the 6 membrane pairs directed the pI markers into the expected separation compartments.

The tunable PVA-based buffering membranes were used in trapping and desalting experiments as both anodic and cathodic membranes. In all of the trapping experiments, small ampholytes were successfully trapped for three hours. In all of the desalting experiments, UV-absorbing salts were successfully removed from a mixture of pI markers.

Lastly, the tunable PVA-based membranes were used as separation membranes in binary IET separations of protein mixtures. First, bovine IgG (approximate molecular weight 150 kDa) was passed through the membrane pores, indicating that the tunable PVA-based membranes were suitable for not only the separation of small compounds, but also large proteins. One membrane from each series was then used as the separation membrane in the modified BF200IET unit to perform a binary protein separation experiment. MAL-PROP-PVA was used to separate the isoforms of ovalbumin from chicken egg white. ASP-PROP-PVA was used to separate the major proteins from chicken egg white. MORPH-ASP-PVA was used to separate the two isoforms of β-lactoglobulin. TRIS-ASP-PVA was used to separate the two major isoforms in horse myoglobin. PROP-ASP-PVA was used to separate the isoforms in hemoglobin, and DEAPA-ASP-PVA was used to separate lysozyme from other major proteins in chicken egg white. All of the tunable PVA-based membranes successfully performed the binary protein separations, and proved to be more hydrolytically stable than the polyacrylamide-based buffering membranes.

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APPENDIX

	A	B	C	D	E	F	G	H
1	**Recipes for MAL-PROP-PVA membranes**							
2			**MW**	**Density**	**Theoretical Mass (g)**	**Amount (mmole)**	**Volume (mL)**	**Mass to weigh out (g)**
3	**PVA paper dimensions**	18 cm x 24 cm						
4	**Number of sheets**	**2**						
5	**Delta pH from pK$_a$**	**0**						
6	**Nominal pK$_a$**	3.8						
7	**MAL/PROP ratio**	0.500						
8	**Crosslink %**	21.000						
9	**Vinyl alcohol/Crosslinker mole ratio**	4.761904762						
10	**Vinyl alcohol/MAL mole ratio**	28						
11	**PVA mass (g) / sheet**	10						
12	**PVA**		44.05		20.000	454.03		**20.000**
13	**NaOH**		40		12.557	313.93		**12.557**
14	**PROP**		75.11	1.012	0.609	8.11		**0.609**
15	**Diethlaminomalonate HCl**		211.65		3.432	16.22		**3.432**
16	**Water (mL/g PVA)**	5	18.02	1.00	100.000	5549.39	100.00	**100.000**
17	**Total mass in flask (g)**				136.598			
18	**Theoretical total GDGE**		204.22	1.229	19.472	95.35	15.84	
19	**Reaction mixture (g) / sheet**							**45.000**
20	**GDGE mass (g) / sheet**							**6.415**
21								
22	**Formulas:**							
23	B7 = 1/(1+10^(-$B$5))			F14 = $B$7*$F$15				
24	B9 = 1*100/$B$8			F15 = $F$12/$B$10				
25	E12 = $B$4*$B$11			F16 = $E$16/$C$16*1000				
26	E13 = $F$13*$C$13/1000			F18 = $F12/$B$9				
27	E14 = $F$14*$C$14/1000			G16 = $E$16/$D$16				
28	E15 = $F$15*$C$15/1000			G18 = $E$18/$D$18				
29	E16 = $E$12*$B$16			H12 = $B$4*$B$11				
30	E17 = SUM($E$12:$E$16)			H13 = $F$13*$C$13/1000				
31	E18 = $C$18*$F$18/1000			H14 = $F$14*$C$14/1000				
32	F12 = 1000*$E$12/$C$12			H15 = $F$15*$C$15/1000				
33	F13 = ($F$12/5+$F$18*2+$F$15*2)			H16 = $E$12*$B$16				
34				H20 = $H19*$E$18/$E$17				

Figure A-1. Excel spreadsheet used to calculate the recipes for the MAL-PROP-PVA membranes.

	A	B	C	D	E	F	G	H
1	**Recipes for ASP-PROP-PVA membranes**							
2			**MW**	**Density**	**Theoretical Mass (g)**	**Amount (mmole)**	**Volume (mL)**	**Mass to weigh out (g)**
3	**PVA paper dimensions**	18 cm x 24 cm						
4	**Number of sheets**	**2**						
5	**Delta pH from pK$_a$**	**0**						
6	**Nominal pK$_a$**	4.7						
7	**ASP/PROP ratio**	0.500						
8	**Crosslink %**	21.000						
9	**Vinyl alcohol/Crosslinker mole ratio**	4.761904762						
10	**Vinyl alcohol/ASP mole ratio**	28						
11	**PVA mass (g) / sheet**	10						
12	**PVA**		44.05		20.000	454.03		**20.000**
13	**NaOH**		40		12.557	313.93		**12.557**
14	**PROP**		75.11	1.012	0.609	8.11		**0.609**
15	**ASP**		133.1		2.158	16.22		**2.158**
16	**Water (mL/g PVA)**	5	18.02	1.00	100.000	5549.39	100.00	**100.000**
17	**Total mass in flask (g)**				135.324			
18	**Theoretical total GDGE**		204.22	1.229	19.472	95.35	15.84	
19	**Reaction mixture (g) / sheet**							**45.000**
20	**GDGE mass (g) / sheet**							**6.475**
21								
22	**Formulas:**							
23	B7 = 1/(1+10^(-$B$5))			F14 = $B$7*$F$15				
24	B9 = 1*100/$B$8			F15 = $F$12/$B$10				
25	E12 = $B$4*$B$11			F16 = $E$16/$C$16*1000				
26	E13 = $F$13*$C$13/1000			F18 = $F12/$B$9				
27	E14 = $F$14*$C$14/1000			G16 = $E$16/$D$16				
28	E15 = $F$15*$C$15/1000			G18 = $E$18/$D$18				
29	E16 = $E12*$B$16			H12 = $B$4*$B$11				
30	E17 = SUM($E$12:$E$16)			H13 = $F$13*$C$13/1000				
31	E18 = $C$18*$F$18/1000			H14 = $F$14*$C$14/1000				
32	F12 = 1000*$E$12/$C$12			H15 = $F$15*$C$15/1000				
33	F13 = ($F$12/5+$F$18*2+$F$15*2)			H16 = $E$12*$B$16				
34				H20 = $H19*$E$18/$E$17				

Figure A-2. Excel spreadsheet used to calculate the recipes for the ASP-PROP-PVA membranes.

	A	B	C	D	E	F	G	H
1	**Recipes for MORPH-ASP-PVA membranes (lower pK_a)**							
2			**MW**	**Density**	**Theoretical Mass (g)**	**Amount (mmole)**	**Volume (mL)**	**Mass to weigh out (g)**
3	**PVA paper dimensions**	18 cm x 24 cm						
4	**Number of sheets**	**2**						
5	**Delta pH from pK_a**	**0**						
6	**Nominal pK_a**	5.6						
7	**MORPH/ASP ratio**	0.667						
8	**Crosslink %**	21.000						
9	**Vinyl alcohol/Crosslinker mole ratio**	4.761904762						
10	**Vinyl alcohol/MORPH mole ratio**	28						
11	**PVA mass (g) / sheet**	10						
12	**PVA**		44.05		20.000	454.03		**20.000**
13	**NaOH**		40		13.206	330.14		**13.206**
14	**MORPH**		130.19	1	2.111	16.22		**2.111**
15	**ASP**		133.1		3.237	24.32		**3.237**
16	**Water (mL/g PVA)**	5	18.02	1.00	100.000	5549.39	100.00	**100.000**
17	**Total mass in flask (g)**				138.554			
18	**Theoretical total GDGE**		204.22	1.229	19.472	95.35	15.84	
19	**Reaction mixture (g) / sheet**							**45.000**
20	**GDGE mass (g) / sheet**							**6.324**
21								
22	**Formulas:**							
23	B7 = 1/(2-1/(1+10^(-$B$5)))			F14 = $F$12/$B$10				
24	B9 = 1*100/$B$8			F15 = $F$14/$B$7				
25	E12 = $B$4*$B$11			F16 = $E$16/$C$16*1000				
26	E13 = $F$13*$C$13/1000			F18 = $F12/$B$9				
27	E14 = $F$14*$C$14/1000			G16 = $E$16/$D$16				
28	E15 = $F$15*$C$15/1000			G18 = $E$18/$D$18				
29	E16 = $E$12*$B$16			H12 = $B$4*$B$11				
30	E17 = SUM($E$12:$E$16)			H13 = $F$13*$C$13/1000				
31	E18 = $C$18*$F$18/1000			H14 = $F$14*$C$14/1000				
32	F12 = 1000*$E$12/$C$12			H15 = $F$15*$C$15/1000				
33	F13 = ($F$12/5+$F$18*2+$F$15*2)			H16 = $E$12*$B$16				
34				H20 = $H19*$E$18/$E$17				

Figure A-3. Excel spreadsheet used to calculate the recipes for the MORPH-ASP-PVA membranes buffering on the lower pK_a in the MORPH-ASP mixture.

	A	B	C	D	E	F	G	H
1	**Recipes for MORPH-ASP-PVA membranes (higher pK_a)**							
2			**MW**	**Density**	**Theoretical Mass (g)**	**Amount (mmole)**	**Volume (mL)**	**Mass to weigh out (g)**
3	**PVA paper dimensions**	18 cm x 24 cm						
4	**Number of sheets**	**2**						
5	**Delta pH from pK_a**	**0**						
6	**Nominal pK_a**	7.6						
7	**MORPH/ASP ratio**	2.000						
8	**Crosslink %**	21.000						
9	**Vinyl alcohol/Crosslinker mole ratio**	4.761904762						
10	**Vinyl alcohol/MORPH mole ratio**	28						
11	**PVA mass (g) / sheet**	10						
12	**PVA**		44.05		20.000	454.03		**20.000**
13	**NaOH**		40		11.909	297.71		**11.909**
14	**MORPH**		130.19	1	2.111	16.22		**2.111**
15	**ASP**		133.1		1.079	8.11		**1.079**
16	**Water (mL/g PVA)**	5	18.02	1.00	100.000	5549.39	100.00	**100.000**
17	**Total mass in flask (g)**				135.099			
18	**Theoretical total GDGE**		204.22	1.229	19.472	95.35	15.84	
19	**Reaction mixture (g) / sheet**							**45.000**
20	**GDGE mass (g) / sheet**							**6.486**
21								
22	Formulas:							
23	B7 = 1/(1-1/(1+10^(-$B$5)))			F14 = $F$12/$B$10				
24	B9 = 1*100/$B$8			F15 = $F$14/$B$7				
25	E12 = $B$4*$B$11			F16 = $E$16/$C$16*1000				
26	E13 = $F$13*$C$13/1000			F18 = $F12/$B$9				
27	E14 = $F$14*$C$14/1000			G16 = $E$16/$D$16				
28	E15 = $F$15*$C$15/1000			G18 = $E$18/$D$18				
29	E16 = $E$12*$B$16			H12 = $B$4*$B$11				
30	E17 = SUM($E$12:$E$16)			H13 = $F$13*$C$13/1000				
31	E18 = $C$18*$F$18/1000			H14 = $F$14*$C$14/1000				
32	F12 = 1000*$E$12/$C$12			H15 = $F$15*$C$15/1000				
33	F13 = ($F$12/5+$F$18*2+$F$15*2)			H16 = $E$12*$B$16				
34				H20 = $H19*$E$18/$E$17				

Figure A-4. Excel spreadsheet used to calculate the recipes for the MORPH-ASP-PVA membranes buffering on the higher pK_a in the MORPH-ASP mixture.

	A	B	C	D	E	F	G	H
1	**Recipes for TRIS-ASP-PVA membranes**							
2			**MW**	**Density**	**Theoretical Mass (g)**	**Amount (mmole)**	**Volume (mL)**	**Mass to weigh out (g)**
3	**PVA paper dimensions**	18 cm x 24 cm						
4	**Number of sheets**	**2**						
5	**Delta pH from pK$_a$**	**0**						
6	**Nominal pK$_a$**	6.5						
7	**TRIS/ASP ratio**	2.000						
8	**Crosslink %**	21.000						
9	**Vinyl alcohol/Crosslinker mole ratio**	4.761904762						
10	**Vinyl alcohol/TRIS mole ratio**	28						
11	**PVA mass (g) / sheet**	10						
12	**PVA**		44.05		20.000	454.03		**20.000**
13	**NaOH**		40		11.909	297.71		**11.909**
14	**TRIS**		121.14		1.964	16.22		**1.964**
15	**ASP**		133.1		1.079	8.11		**1.079**
16	**Water (mL/g PVA)**	5	18.02	1.00	100.000	5549.39	100.00	**100.000**
17	**Total mass in flask (g)**				134.952			
18	**Theoretical total GDGE**		204.22	1.229	19.472	95.35	15.84	
19	**Reaction mixture (g) / sheet**							**45.000**
20	**GDGE mass (g) / sheet**							**6.493**
21								
22	Formulas:							
23	B7 = 1/(1-1/(1+10^(-$B$5)))			F14 = $F$12/$B$10				
24	B9 = 1*100/$B$8			F15 = $F$14/$B$7				
25	E12 = $B$4*$B$11			F16 = $E$16/$C$16*1000				
26	E13 = $F$13*$C$13/1000			F18 = $F12/$B$9				
27	E14 = $F$14*$C$14/1000			G16 = $E$16/$D$16				
28	E15 = $F$15*$C$15/1000			G18 = $E$18/$D$18				
29	E16 = $E$12*$B$16			H12 = $B$4*$B$11				
30	E17 = SUM($E$12:$E$16)			H13 = $F$13*$C$13/1000				
31	E18 = $C$18*$F$18/1000			H14 = $F$14*$C$14/1000				
32	F12 = 1000*$E$12/$C$12			H15 = $F$15*$C$15/1000				
33	F13 = ($F$12/5+$F$18*2+$F$15*2)			H16 = $E$12*$B$16				
34				H20 = $H19*$E$18/$E$17				

Figure A-5. Excel spreadsheet used to calculate the recipes for the TRIS-ASP-PVA membranes.

	A	B	C	D	E	F	G	H
1	**Recipes for PROP-ASP-PVA membranes**							
2			**MW**	**Density**	**Theoretical Mass (g)**	**Amount (mmole)**	**Volume (mL)**	**Mass to weigh out (g)**
3	**PVA paper dimensions**	18 cm x 24 cm						
4	**Number of sheets**	**2**						
5	**Delta pH from pK$_a$**	**0**						
6	**Nominal pK$_a$**	7.5						
7	**PROP/ASP ratio**	2.000						
8	**Crosslink %**	21.000						
9	**Vinyl alcohol/Crosslinker mole ratio**	4.761904762						
10	**Vinyl alcohol/PROP mole ratio**	28						
11	**PVA mass (g) / sheet**	10						
12	**PVA**		44.05		20.000	454.03		**20.000**
13	**NaOH**		40		11.909	297.71		**11.909**
14	**PROP**		75.11	1.012	1.218	16.22		**1.218**
15	**ASP**		133.1		1.079	8.11		**1.079**
16	**Water (mL/g PVA)**	5	18.02	1.00	100.000	5549.39	100.00	**100.000**
17	**Total mass in flask (g)**				134.206			
18	**Theoretical total GDGE**		204.22	1.229	19.472	95.35	15.84	
19	**Reaction mixture (g) / sheet**							**45.000**
20	**GDGE mass (g) / sheet**							**6.529**
21								
22	**Formulas:**							
23	B7 = 1/(1-1/(1+10^(-$B$5)))			F14 = $F$12/$B$10				
24	B9 = 1*100/$B$8			F15 = $F$14/$B$7				
25	E12 = $B$4*$B$11			F16 = $E$16/$C$16*1000				
26	E13 = $F$13*$C$13/1000			F18 = $F12/$B$9				
27	E14 = $F$14*$C$14/1000			G16 = $E$16/$D$16				
28	E15 = $F$15*$C$15/1000			G18 = $E$18/$D$18				
29	E16 = $E$12*$B$16			H12 = $B$4*$B$11				
30	E17 = SUM($E$12:$E$16)			H13 = $F$13*$C$13/1000				
31	E18 = $C$18*$F$18/1000			H14 = $F$14*$C$14/1000				
32	F12 = 1000*$E$12/$C$12			H15 = $F$15*$C$15/1000				
33	F13 = ($F$12/5+$F$18*2+$F$15*2)			H16 = $E$12*$B$16				
34				H20 = $H19*$E$18/$E$17				

Figure A-6. Excel spreadsheet used to calculate the recipes for the PROP-ASP-PVA membranes.

	A	B	C	D	E	F	G	H
1	**Recipes for DEAPA-ASP-PVA membranes (lower pK_a)**							
2			**MW**	**Density**	**Theoretical Mass (g)**	**Amount (mmole)**	**Volume (mL)**	**Mass to weigh out (g)**
3	**PVA paper dimensions**	18 cm x 24 cm						
4	**Number of sheets**	**2**						
5	**Delta pH from pK_a**	**0**						
6	**Nominal pK_a**	7.6						
7	**DEAPA/ASP ratio**	0.667						
8	**Crosslink %**	21.000						
9	**Vinyl alcohol/Crosslinker mole ratio**	4.761904762						
10	**Vinyl alcohol/DEAPA mole ratio**	28						
11	**PVA mass (g) / sheet**	10						
12	**PVA**		44.05		20.000	454.03		**20.000**
13	**NaOH**		40		13.206	330.14		**13.206**
14	**DEAPA**		130.23	0.826	2.112	16.22		**2.112**
15	**ASP**		133.1		3.237	24.32		**3.237**
16	**Water (mL/g PVA)**	5	18.02	1.00	100.000	5549.39	100.00	**100.000**
17	**Total mass in flask (g)**				138.555			
18	**Theoretical total GDGE**		204.22	1.229	19.472	95.35	15.84	
19	**Reaction mixture (g) / sheet**							**45.000**
20	**GDGE mass (g) / sheet**							**6.324**
21								
22	**Formulas:**			F14 = $F$12/$B$10				
23	B7 = 1/(2-1/(1+10^(-$B$5)))			F15 = $F$14/$B$7				
24	B9 = 1*100/$B$8			F16 = $E$16/$C$16*1000				
25	E12 = $B$4*$B$11			F18 = $F12/$B$9				
26	E13 = $F$13*$C$13/1000			G16 = $E$16/$D$16				
27	E14 = $F$14*$C$14/1000			G18 = $E$18/$D$18				
28	E15 = $F$15*$C$15/1000			H12 = $B$4*$B$11				
29	E16 = $E$12*$B$16			H13 = $F$13*$C$13/1000				
30	E17 = SUM($E$12:$E$16)			H14 = $F$14*$C$14/1000				
31	E18 = $C$18*$F$18/1000			H15 = $F$15*$C$15/1000				
32	F12 = 1000*$E$12/$C$12			H16 = $E$12*$B$16				
33	F13 = ($F$12/5+$F$18*2+$F$15*2)			H20 = $H19*$E$18/$E$17				
34								

Figure A-7. Excel spreadsheet used to calculate the recipes for the DEAPA-ASP-PVA membranes buffering on the lower pK_a in the DEAPA-ASP mixture.

	A	B	C	D	E	F	G	H
1	**Recipes for DEAPA-ASP-PVA membranes (higher pK_a)**							
2			**MW**	**Density**	**Theoretical Mass (g)**	**Amount (mmole)**	**Volume (mL)**	**Mass to weigh out (g)**
3	**PVA paper dimensions**	18 cm x 24 cm						
4	**Number of sheets**	**2**						
5	**Delta pH from pK_a**	**0**						
6	**Nominal pK_a**	8.9						
7	**DEAPA/ASP ratio**	2.000						
8	**Crosslink %**	21.000						
9	**Vinyl alcohol/Crosslinker mole ratio**	4.761904762						
10	**Vinyl alcohol/DEAPA mole ratio**	28						
11	**PVA mass (g) / sheet**	10						
12	**PVA**		44.05		20.000	454.03		**20.000**
13	**NaOH**		40		11.909	297.71		**11.909**
14	**DEAPA**		130.23	0.826	2.112	16.22		**2.112**
15	**ASP**		133.1		1.079	8.11		**1.079**
16	**Water (mL/g PVA)**	5	18.02	1.00	100.000	5549.39	100.00	**100.000**
17	**Total mass in flask (g)**				135.099			
18	**Theoretical total GDGE**		204.22	1.229	19.472	95.35	15.84	
19	**Reaction mixture (g) / sheet**							**45.000**
20	**GDGE mass (g) / sheet**							**6.486**
21								
22	**Formulas:**							
23	B7 = 1/(1-1/(1+10^(-$B$5)))			F14 = $F$12/$B$10				
24	B9 = 1*100/$B$8			F15 = $F$14/$B$7				
25	E12 = $B$4*$B$11			F16 = $E$16/$C$16*1000				
26	E13 = $F$13*$C$13/1000			F18 = $F12/$B$9				
27	E14 = $F$14*$C$14/1000			G16 = $E$16/$D$16				
28	E15 = $F$15*$C$15/1000			G18 = $E$18/$D$18				
29	E16 = $E$12*$B$16			H12 = $B$4*$B$11				
30	E17 = SUM($E$12:$E$16)			H13 = $F$13*$C$13/1000				
31	E18 = $C$18*$F$18/1000			H14 = $F$14*$C$14/1000				
32	F12 = 1000*$E$12/$C$12			H15 = $F$15*$C$15/1000				
33	F13 = ($F$12/5+$F$18*2+$F$15*2)			H16 = $E$12*$B$16				
34				H20 = $H19*$E$18/$E$17				

Figure A-8. Excel spreadsheet used to calculate the recipes for the DEAPA-ASP-PVA membranes buffering on the higher pK_a in the DEAPA-ASP mixture.

VITA

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